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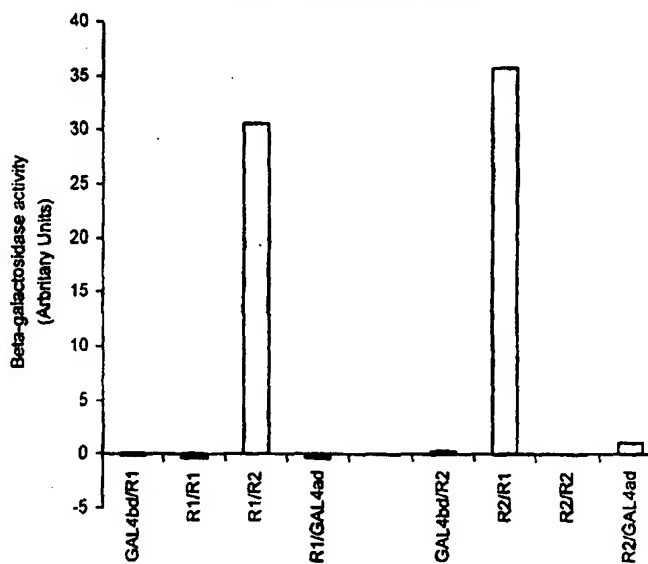
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(54) Title: GABAB RECEPTOR SUBTYPES GABAB-R1c AND GABAB-R2 AND HETERODIMERS THEREOF

(57) Abstract

The present invention relates to the novel GABAB receptor subtypes GABAB-R1c and GABAB-R2 as well as to a novel, functional GABAB receptor which comprises a heterodimer of GABAB-R1 and GABAB-R2 receptor subunits. The present invention also relates to variant of the receptors, nucleotide sequences encoding the receptors and variants thereof and novel vectors, stable cell lines, antibodies, screening methods, methods of treatment and methods of receptor production.

HETERODIMERSATION AND HOMODIMERISATION BETWEEN THE C-TERMINAL DOMAINS OF THE GABAB-R1 AND GABAB-R2 RECEPTORS IN THE YEAST TWO HYBRID SYSTEM.

GABAB RECEPTOR SUBTYPES GABAB–R1c AND GABAB–R2 AND HETERODIMERS THEREOF

Field of the Invention

5   The present invention relates to the novel $GABA_B$ receptor subtypes $GABA_B$-R1c and $GABA_B$-R2 as well as to a novel, functional $GABA_B$ receptor which comprises a heterodimer of $GABA_B$-R1 and $GABA_B$-R2 receptor subunits. The present invention also relates to variants of the receptors, nucleotide sequences encoding the receptors and variants thereof and novel vectors, stable cell lines,

10   antibodies, screening methods, methods of treatment and methods of receptor production.

Background of the Invention

GABA (γ-amino-butyric acid) is the main inhibitory neurotransmitter in the central

15   nervous system (CNS) activating two distinct families of receptors; the ionotropic $GABA_A$ and $GABA_C$ receptors for fast synaptic transmissions, and the metabotropic $GABA_B$ receptors governing a slower synaptic transmission. $GABA_B$ receptors are members of the superfamily of 7-transmembrane G protein-coupled receptors. Activation results in signal transduction through a

20   variety of pathways mediated principally via members of the G_i/G_o family of pertussis toxin-sensitive G proteins. $GABA_B$ receptors have been shown to inhibit N, P/Q and T-type Ca^{2+} channels in a pertussis toxin-sensitive manner (Kobrinsky *et al.*, 1993; Menon-Johansson *et al.*, 1993; Harayama *et al.*, 1998) and indeed there is also some evidence for direct interactions between $GABA_B$

25   receptors and Ca^{2+} channels since Ca^{2+} channel ligands can modify the binding of $GABA_B$ agonists (Ohmori *et al.*, 1990). $GABA_B$ receptor-mediated Ca^{2+} channel inhibition is the principle mechanism for presynaptic inhibition of neurotransmitter release. Post-synaptically the major effect of $GABA_B$ receptor activation is to open potassium channels, to generate post-synaptic inhibitory

30   potentials.

Autoradiographic studies show that $GABA_B$ receptors are abundant and heterogeneously distributed throughout the CNS, with particularly high levels in the molecular layer of the cerebellum, interpeduncular nucleus, frontal cortex,

35   olfactory nuclei and thalamic nuclei. $GABA_B$ receptors are also widespread in the

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globus pallidus, temporal cortex, raphe magnus and spinal cord (Bowery et al., 1987). $GABA_B$ receptors are an important therapeutic target in the CNS for conditions such as spasticity, epilepsy, Alzheimer's disease, pain, affective disorders and feeding. $GABA_B$ receptors are also present in the peripheral

5   nervous system, both on sensory nerves and on parasympathetic nerves. Their ability to modulate these nerves gives them potential as targets in disorders of the lung, GI tract and bladder (Kerr and Ong, 1995; 1996; Malcangio and Bowery, 1995).

10   Despite the widespread abundance of $GABA_B$ receptors, considerable evidence from neurochemical, electrophysiological and behavioural studies suggests that multiple subtypes of $GABA_B$ receptors exist. This heterogeneity of $GABA_B$ receptors may allow the development of selective ligands, able to target specific aspects of $GABA_B$ receptor function. This would lead to the development of

15   drugs with improved selectivity profiles relative to current compounds (such as baclofen) which are relatively non-selective and show a variety of undesirable behavioural actions such as sedation and respiratory depression. Multiple receptor subtypes are best classified by the differing profiles of agonist and antagonist ligands.

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To date screening for $GABA_B$ ligands and subsequent structure / activity determinations has relied on radioligand binding assays to rat brain membranes. Further analysis of such ligands in animal models has indicated differences in their behavioural profile. However, due to the absence of cloned $GABA_B$

25   receptors the molecular basis for such differences has not been defined, and therefore it has not been possible to optimise $GABA_B$ ligands for therapeutic use.

$GABA_B$ receptors were first described nearly 20 years ago (Hill and Bowery, 1981), but despite extensive efforts using conventional expression cloning

30   strategies, for example in Xenopus oocytes, or cloning based on sequence homology, the molecular nature of the $GABA_B$ receptor remained elusive. The development of a high affinity antagonist for the receptor finally allowed Kaupmann et al., (1997) to expression clone the receptor from a rat cerebral cortex cDNA using a radioligand binding assay. Two splice variants of the

35   receptor were identified, $GABA_B$-R1a encoding a 960 amino acid protein and

GABA$_B$-R1b, encoding an 844 amino acid protein, differing only in the lengths of their N-termini. These two splice variants have distinct spatial distributions within the brain, but both reside within neuronal rather than glial cells. Pharmacologically, the two splice variants are similar, showing binding affinities

5   for a range of antagonists, but about 10 fold lower than those of native receptors, as well as agonist displacement constants which are about 100-150 fold lower than those of native receptors. These observations have led to speculation that the cloned receptor was a low affinity receptor and an additional high affinity, pharmacologically distinct GABA$_B$ receptor subtype could exist in

10   the brain. Alternatively, it was argued that G-protein coupling was inefficient or the receptor was desensitising in the recombinant systems used.

A number of groups working in the area have, however, found that the cloned receptor fails to behave as a functional GABA$_B$ receptor either in mammalian

15   cells or in *Xenopus* oocytes. The present invention describes the cloning of a novel human GABA$_B$ receptor subtype, GABA$_B$-R2, the identification of a novel splice variant GABA$_B$-R1c, and the surprising observation that GABA$_B$-R1 and GABA$_B$-R2 strongly interact via their C-termini to form heterodimers. Co-expression of GABA$_B$-R1 and GABA$_B$-R2 allows trafficking of GABA$_B$-R1 to the

20   cell surface and results in a high affinity functional GABA$_B$ receptor in both mammalian cells and *Xenopus* oocytes.

These surpising findings provide a unique opportunity to define GABA$_B$ subtypes at the molecular level, which in turn will lead to the identification of novel

25   subtype-specific drugs.

Summary of the Invention
According to one embodiment of the present invention there is provided an isolated GABA$_B$-R2 receptor protein or a variant thereof.

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According to another embodiment of the invention there is provided an isolated GABA$_B$-R2 receptor protein having amino acid sequence provided in Fig. 1B, or a variant thereof.

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According to a further embodiment of the invention there is provided a nucleotide sequence encoding a $GABA_B$-R2 receptor or a variant thereof, or a nucleotide sequence which is complementary thereto.

5   According to a further embodiment of the invention there is provided a nucleotide sequence encoding a $GABA_B$-R2 receptor, as shown in Fig. 1A, or a variant thereof, or a nucleotide sequence which is complementary thereto.

According to a further embodiment of the invention there is provided an
10   expression vector comprising a nucleotide sequence as referred to above which is capable of expressing a $GABA_B$-R2 receptor protein or a variant thereof.

According to a still further embodiment of the invention there is provided a stable cell line comprising a vector as referred to above.

15
According to another embodiment of the invention there is provided an antibody specific for a $GABA_B$-R2 receptor protein or a variant thereof.

According to another embodiment of the invention there is provided an isolated
20   $GABA_B$-R1c receptor protein or a variant thereof.

According to another embodiment of the invention there is provided an isolated $GABA_B$-R1c receptor protein having amino acid sequence provided in Fig. 2, or a variant thereof.
25
According to another embodiment of the invention there is provided a nucleotide sequence encoding a $GABA_B$-R1c receptor protein or a variant thereof, or a nucleotide sequence which is complementary thereto.

30   According to another embodiment of the invention there is provided an expression vector comprising a nucleotide sequence as referred to above, which is capable of expressing a $GABA_B$-R1c receptor protein or a variant thereof.

According to another embodiment of the invention there is provided a stable cell
35   line comprising a vector as referred to above.

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According to a further embodiment of the invention there is provided an antibody specific for a GABA$_B$-R1c receptor protein or a variant thereof.

5   According to a further embodiment of the invention there is provided a GABA$_B$ receptor comprising an heterodimer between a GABA$_B$-R1 receptor protein or a variant thereof and a GABA$_B$-R2 receptor protein or a variant thereof.

According to a further embodiment of the invention there is provided an
10   expression vector comprising a nucleotide sequence encoding for a GABA$_B$-R1 receptor or a variant thereof and a nucleotide sequence encoding for a GABA$_B$-R2 receptor or variant thereof, said vector being capable of expressing both GABA$_B$-R1 and GABA$_B$-R2 receptor proteins or variants thereof.

15   According to a further embodiment of the invention there is provided a stable cell line comprising a vector as referred to above.

According to a further embodiment of the invention there is provided a stable cell line modified to express both GABA$_B$-R1 and GABA$_B$-R2 receptor proteins or
20   variants thereof.

According to a further embodiment of the invention there is provided a GABA$_B$ receptor produced by a stable cell line as referred to above.

25   According to a further embodiment of the invention there is provided an antibody specific for a GABA$_B$ receptor as referred to above.

According to a further embodiment of the invention there is provided a method for identification of a compound which exhibits GABA$_B$ receptor modulating
30   activity, comprising contacting a GABA$_B$ receptor as referred to above with a test compound and detecting modulating activity or inactivity.

According to a further embodiment of the invention there is provided a compound which modulates GABA$_B$ receptor activity, identifiable by a method as
35   referred to above.

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According to a further embodiment of the invention there is provided a method of treatment or prophylaxis of a disorder which is responsive to modulation of GABA$_B$ receptor activity in a mammal, which comprises administering to said mammal an effective amount of a compound identifiable by the method referred to above.

Brief Description of the Figures

Figure 1. Nucleotide and protein sequences of Human GABA$_B$-R2
Nucleotide sequence (a) and the translated protein sequence (b) for Human GABA$_B$-R2 are shown.

Figure 2. Protein alignments between GABA$_B$-R1a, GABA$_B$-R1b, GABA$_B$-R1c splice variants and GABA$_B$-R2.
Amino-acid sequences of the human GABA$_B$-R1a, GABA$_B$-R1b and GABA$_B$-R2 receptors aligned for comparison. Signal sequences and predicted cleavage point ($\times$), together with the N-terminal splice points for GABA$_B$-R1a and GABA$_B$-R1b are shown. GABA$_B$-R1c sequence is exactly that of GABA$_B$-R1a, except for the deletion of 63 amino acids (open box). Amino acids conserved between GABA$_B$-R1a and GABA$_B$-R1b are in bold type and potential *N*-glycosylation sites (*) are shown. Lines beneath the text show positions of the seven predicted TM domains and regions encoding coiled coil structure are indicated by shading. The C-terminal region of GABA$_B$-R1 used as the bait in the yeast two hybrid analysis is marked as 'BAIT→', and GABA$_B$-R2 C-terminal domains recovered from the library screen against GABA$_B$-R1 C-terminus are shown as 'YTH HITS→'.

Figure 3. Hydrophobicity profile of GABA$_B$-R2.
Hydrophobicity profiles of GABA$_B$-R2 sequence were determined using the Kyte-Doolittle algorithm, whereby positive values indicate hydrophobic regions. The predicted signal sequence and seven trans-membrane domains are shown.

Figure 4. Tissue Distribution Studies for Human GABA$_B$-R1 and GABA$_B$-R2.
A Human RNA Master Blot (Clontech), containing normalised polyA$^+$ mRNA from multiple tissues of adult and fetal origin, were probed sequentially with a pan

specific probe for GABA$_B$-R1 (all splice variants) followed by a GABA$_B$-R2 specific probe. Resulting autoradiographic analysis of the blots are shown, together with a grid identifying tissue type. Specificity controls include yeast RNA and *E. coli* DNA.

Figure 5. Heterodimerisation and homodimerisation between the C-terminal domains of the GABA$_B$-R1 and GABA$_B$-R2 receptors in the yeast two hybrid system.

β-galactosidase activity was measured in yeast Y190 cells expressing the GABA$_B$-R1 or the GABA$_B$-R2 C-termini, either against empty vector or against each other in all combinations, using ONPG. Of each pair of proteins expressed in the two hybrid system, the first always refers to the GAL4$_{BD}$ fusion construct whilst the second refers to the GAL4$_{AD}$ fusion construct. β-galactosidase activity is determined relative to cell numbers and is in arbitary units.

Figure 6. Co-immunoprecipitation studies of the GABA$_B$ heterodimer in HEK239 cells.

HEK293T cells were transfected with 1 μg each of either Myc-GABA$_B$-R1b or HA-GABA$_B$-R2 alone or in combination. Cells were harvested 48 h after transfection, lysed and epitope tagged receptors immunoprecipitated using 12CA5 (HA) or 9E10 (Myc) antisera as described in Methods. Immune complexes were then subjected to SDS-PAGE, transferred to nitrocellulose, and captured Myc-GABA$_B$-R1b and HA-GABA$_B$-R2 identified by immunoblotting with Myc and HA, respectively. Lanes 1 and 4, immunoprecipitates of cells transfected with Myc-GABA$_B$-R1b only; lanes 2 and 5, HA-GABA$_B$-R2 only; lanes 3 and 6, immunoprecipitates of cells transfected with Myc-GABA$_B$-R1b together with HA-GABA$_B$-R2. Lanes 1-3, lysates immunoprecipitated with 9E10 (Myc) and blotted to 12CA5(HA); lanes 4-6, lysates immunoprecipitated with 12CA5(HA) and blotted with 9E10 (Myc)

Figure 7. Cell surface localisation of GABA$_B$-R1 receptor is dependent upon coexpression with GABA$_B$-R2.

Flow cytometry was performed on HEK293T cells transfected with 1μg of either Myc-GABA$_B$-R1b or HA-GABA$_B$-R2 or both receptors in combination. **(A)** Analysis using 9E10 (c-Myc) as primary antibody to detect Myc-GABA$_B$-R1b;

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intact cells. **(B)** Analysis using 9E10 (c-Myc) as primary antibody to detect Myc-GABA$_B$-R1b; permeabilised cells. **(C)** Analysis using 12CA5 (HA) as primary antibody to detect HA-GABA$_B$-R2; intact cells. Mock transfected cells, reflecting background fluorescence, are shaded and the marker indicates fluorescence measured over background levels. Myc-GABA$_B$-R1b data is shown as a grey line whereas co-expression of Myc-GABA$_B$-R1b with HA-GABA$_B$-R2 is shown in black. 30,000 cells were analysed in each sample. Histograms shown are from a single experiment. Quoted statistics are from mean of three separate transfections and analysis.

Figure 8. Coexpression of GABA$_B$-R1a and 1b splice variants with GABA$_B$-R2 receptors in HEK293T cells results in terminal glycosylation of both GABA$_B$-R1a and GABA$_B$-R1b.

P2 membrane fractions were derived from HEK293T cells that were transfected with 1 µg of either GABA$_B$-R1a (lanes 1-3), GABA$_B$-R1b (lanes 4-6) or HA-GABA$_B$-R2 (lanes 13-15), or with 1 µg each of HA-GABA$_B$-R2 in combination with 1 µg of either GABA$_B$-R1a (lanes 7-9, 16-18) or GABA$_B$-R1b (lanes 10-12, 19-21). Glycosylation status of transfected receptors was assessed following treatment of P2 fractions (50 µg of membrane protein) with either vehicle (lanes 1, 4, 7, 10, 13, 16 and 19), endoglycosidase F (lanes 2, 5, 8, 11, 14, 17 and 20) or endoglycosidase H (lanes 3, 6, 9, 12, 15, 18 and 21). Samples were resolved by SDS-PAGE (10 % (w/v) acrylamide), transferred to nitrocellulose, and immunoblotted. Upper panel, antiserum 501 was used as primary reagent to allow identification of both GABA$_B$-R1a and 1b. Lower panel, 12CA5 anti-HA antiserum was employed to identify HA-GABA$_B$-R2. *, denotes terminally glycosylated forms of GABA$_B$-R1a and 1b.

Figure 9. Coexpression of GABA$_B$-R1 and GABA$_B$-R2 receptors in HEK293T cells leads to GABA-mediated stimulation of [^{35}S]GTPγS binding activity.

[^{35}S]GTPγS binding activity was measured on P2 particulate fractions derived from HEK293T cells transfected with 1 µg of G$_{o1}$α together with 1 µg of either GABA$_B$-R1a, GABA$_B$-R1b or HA-GABA$_B$-R2; or with 1 µg each of G$_{o1}$α and HA-GABA$_B$-R2 in combination with 1 µg of either GABA$_B$-R1a or GABA$_B$-R1b. **(A)** [^{35}S]GTPγS binding was measured in the absence (open bars) or presence (hatched bars) of GABA (10 mM) as described in Methods. **(B)** The ability of

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varying concentrations of GABA to stimulate the binding of $[^{35}S]GTP\gamma S$ was measured on P2 membrane fractions from HEK293T cells expressing either $G_{o1}\alpha$ and HA-GABA$_B$-R2 alone (open circles) or in combination with either GABA$_B$-R1a (closed squares) or GABA$_B$-R1b (closed triangles). The data shown are the means ± S.D. of triplicate measurements and are representative of three independent experiments.

Figure 10. GABA-mediated stimulation of $[^{35}S]GTP\gamma S$ binding activity in HEK293T cells coexpressing GABA$_B$-R1 and GABA$_B$-R2 receptors requires cotransfection with additional G$_i$ G protein, $G_{o1}\alpha$.

$[^{35}S]GTP\gamma S$ binding activity was measured on P2 particulate fractions derived from HEK293T cells transfected with HA-GABA$_B$-R1b (1μg) together with HA-GABA$_B$-R2 (1μg) and $G_{o1}\alpha$ (1μg) (closed triangles), or in combination with either HA-GABA$_B$-R2 (1μg) (open circles) or $G_{o1}\alpha$ (1μg) (closed circles). The ability of varying concentrations of GABA to stimulate the binding of $[^{35}S]GTP\gamma S$ was determined. Data shown are the mean ± S.D. of triplicate measurements.

Figure 11. Coexpression of GABA$_B$-R1 and GABA$_B$-R2 receptors in HEK293T cells permits GABA-mediated inhibition of forskolin-stimulated adenylate cyclase activity

cAMP levels were measured in HEK293T cells transfected with 1 μg of $G_{i1}\alpha$ together with 1 μg of either GABA$_B$-R1a, GABA$_B$-R1b or HA-GABA$_B$-R2; or with 1 μg each of $G_{i1}\alpha$ and HA-GABA$_B$-R2 in combination with 1 μg of either GABA$_B$-R1a or GABA$_B$-R1b, as described in Methods. **(A)** cAMP levels were determined in cells treated with forskolin (50 μM) in the absence (open bars) or presence (hatched bars) of GABA (1 mM). **(B)** ability of varying concentrations of GABA to inhibit forskolin-elevated adenylate cyclase activity in HEK293T cells expressing $G_{i1}\alpha$ and HA-GABA$_B$-R2 in combination with GABA$_B$-R1b. The data shown are the means ± S.D. of triplicate measurements.

Figure 12. Co-expression of GABA$_B$-R1 and GABA$_B$-R2 receptors in *Xenopus* oocytes permits agonist–dependant activation of ion flux through CFTR and GIRK1/4.

Xenopus oocytes were injected with cRNA encoding GABA$_B$-R1 and GABA$_B$-R2 receptors (in equal amounts for CFTR, 1:2 ratio for GIRK) plus either CFTR (A)

or the GIRK1/GIRK4 heteromer (B). A, Time course plot for an oocyte expressing $GABA_B$-R1, $GABA_B$-R2 and CFTR. Application of 100mM GABA, 100mM SKF97541 or 1mM Baclofen (arrows) activated a large inward CFTR current. Note the increase in CFTR response seen with repeated GABA application. B, Time course plot for an oocyte expressing $GABA_B$-R1, $GABA_B$-R2, GIRK1 and GIRK4. Switching from ND96 (low potassium) to 90K (high potassium) solution led to an inward shift in holding current, showing that the GIRK1/GIRK4 channel is expressed in this oocyte. Subsequent application of 100mM GABA activated a large inward current (middle panel). Negative and positive control experiments are shown from oocytes expressing the $GABA_B$-R2 receptor alone (left panel) and those expressing the adenosine A1 receptor (right panel).

Figure 13. Current-voltage curves in an oocyte expressing $GABA_B$-R1, $GABA_B$-R2 and the potassium channels GIRK1 and GIRK4.
Current-voltage curves are shown for a single oocyte following application of 200ms voltage-clamp pulses from a holding potential of –60mV to test potentials between –100mV and +50mV. Steady-state current is plotted against test potential in ND96 solution (low potassium), 90K solution (90mM potassium) and 90K plus 100mM GABA. Note the basal GIRK1/4 current recorded in 90K solution and the large agonist-evoked activation of the GIRK potassium channel.

Figure 14. GABA-mediated stimulation of [^{35}S]GTPγS binding activity is dependent on the relative levels of expression of $GABA_B$-R1 and $GABA_B$-R2 receptors
HEK293T cells were transfected with HA-$GABA_B$-R2 (1µg) and $G_{o1}\alpha$ (1µg) together with various amounts (0-1 µg) of HA-$GABA_B$-R1b. Cells were harvested 48 h after transfection and P2 membrane fractions were prepared. **(A)** Agonist stimulation of [^{35}S]GTPγS binding activity measured in transfected cell membranes in the presence of GABA (10 mM). Data are shown as stimulation above basal (cpm) and are the mean ± S.D. of triplicate measurements. **(B)** Cell membranes were immunoblotted with anti-HA antiserum to allow the relative levels of HA-$GABA_B$-R2 and HA-$GABA_B$-R1b receptors to be evaluated.

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Figure 15. Co-expression of GABA$_B$-R1 and GABA$_B$-R2 receptors in HEK293T cells generates a high affinity GABA$_B$ binding site similar to brain GABA$_B$ receptors.

P2 membrane fractions were prepared from HEK 293T cells transfected using the same conditions described for GTPγS binding studies. % specific binding was determined for the displacement of [3H]-CGP54626 by GABA. Data shown are the mean of minimum of triplicate studies ± sem.

Detailed Description of the Invention

Throughout the present specification and the accompanying claims the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

As previously explained, the present invention includes a number of important aspects. In particular the present invention relates to isolated GABA$_B$-R2 receptor proteins and variants thereof, isolated GABA$_B$-R1c receptor proteins and variants thereof, GABA$_B$ receptors comprising an heterodimer between a GABA$_B$-R1 receptor protein or a variant thereof and a GABA$_B$-R2 receptor protein or a variant thereof, as well as other related aspects. In the context of the present invention the wording "isolated" is intended to convey that the receptor protein is not in its native state, insofar as it has been purified at least to some extent or has been synthetically produced, for example by recombinant methods. The term "isolated" therefore includes the possibility of the receptor protein being in combination with other biological or non-biological material, such as cells, suspensions of cells or cell fragments, proteins, peptides, organic or inorganic solvents, or other materials where appropriate, but excludes the situation where the receptor protein is in a state as found in nature.

Routine methods, as further explained in the subsequent experimental section, can be employed to purify and/or synthesise the receptor proteins according to the invention. Such methods are well understood by persons skilled in the art, and include techniques such as those disclosed in Sambrook, J. et al, 1989, the disclosure of which is included herein in its entirety by way of reference.

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The present invention not only includes the GABA$_B$ receptor proteins specifically recited, but also variants thereof. By the term "variant" what is meant throughout the specification and claims is that other peptides or proteins which retain the

5   same essential character of the receptor proteins for which sequence information is provided, are also intended to be included within the scope of the invention. For example, other peptides or proteins with greater than about 80%, preferably at least 90% and particularly preferably at least 95% homology with the sequences provided are considered as variants of the receptor proteins.

10   Such variants may include the deletion, modification or addition of single amino acids or groups of amino acids within the protein sequence, as long as the biological functionality of the peptide is not adversely affected.

The invention also includes nucleotide sequences which encode for GABA$_B$-R2

15   or GABA$_B$-R1c receptors or variants thereof as well as nucleotide sequences which are complementary thereto. Preferably the nucleotide sequence is a DNA sequence and most preferably, a cDNA sequence.

The present invention also includes expression vectors which comprise

20   nucleotide sequences encoding for the GABA$_B$-R2 or GABA$_B$-R1c receptor subtypes or variants thereof. A further aspect of the invention relates to an expression vector comprising nucleotide sequences encoding for a GABA$_B$-R1 receptor protein and a GABA$_B$-R2 receptor protein or variants thereof. Such expression vectors are routinely constructed in the art of molecular biology and

25   may involve the use of plasmid DNA and appropriate initiators, promoters, enhancers and other elements, which may be necessary, and which are positioned in the correct orientation, in order to allow for protein expression.

The invention also includes cell lines which have been modified to express the

30   novel receptor. Such cell lines include transient, or preferably stable higher eukaryotic cell lines, such as mammalian cells or insect cells, lower eukaryotic cells, such as yeast or prokaryotic cells such as bacterial cells. Particular examples of cells which have been modified by insertion of vectors encoding for the receptor proteins according to the invention include HEK293T cells and

35   oocytes. Preferably the cell line selected will be one which is not only stable, but

also allows for mature glycosylation and cell surface expression of the inventive receptors. In the case of the functional GABA$_B$ receptor which comprises a heterodimer of GABA$_B$-R1 and GABA$_{-B}$-R2 subunits, the cell line may include a single vector which allows for expression of both of the receptor subtypes, or

5   alternatively separate vectors for each subunit. It is preferred however, that the receptor subtypes should be co-expressed in order to optimise the dimerisation process, which will result in full glycosylation and transport of the glycosylated dimer to the cell surface.

10   It is also possible for the receptors of the invention to be transiently expressed in a cell line or on a membrane, such as for example in a baculovirus expression system. Such systems, which are adapted to express the receptors according to the invention, are also included within the scope of the present invention.

15   A particularly preferred aspect of the invention is the heterodimer formed between the GABA$_B$-R1 and GABA$_B$-R2 receptor proteins which results in the formation of a functional GABA$_B$ receptor. Without wishing to be bound by theory, it appears that the formation of the heterodimer takes place via the coiled-coil domains within the receptor C-terminal tails, and that this in turn is a

20   pre-requisite for transport and full glycosylation of a GABA$_B$-R1, and also for generation of an high affinity GABA$_B$ receptor at the cell surface.

          The heterodimer which forms a functional GABA$_B$ receptor can comprise any GABA$_B$-R1 receptor subtype or splice variant, or variants thereof. Although we

25   are presently only aware of only one GABA$_B$-R2 subtype, it is envisaged that the heterodimers according to the present invention can include other GABA$_B$-R2 subtypes or splice variants which have not yet been identified, as well as variants of the already identified GABA$_B$-R2 receptor proteins.

30   In particular, the functional GABA$_B$ receptor may include GABA$_B$-R1 receptor proteins selected from GABA$_B$-R1a, GABA$_B$-R1b, GABA$_B$-R1c splice variants, variants thereof or even other GABA$_B$-R1 receptor subtypes or splice variants which have not yet been identified.

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According to another aspect, the present invention also relates to antibodies which have been raised by standard techniques and are specific for the receptor proteins or variants thereof according to the invention. Such antibodies could for example, be useful in purification, isolation or screening involving immuno

5   precipitation techniques and may be used as tools to further ellucidate $GABA_B$ receptor function, or indeed as therapeutic agents in their own right. Antibodies may also be raised against specific epitopes of the receptors according to the invention, as opposed to the monomer subunits.

10   An important aspect of the present invention is the use of receptor proteins according to the invention, particularly the heterodimer $GABA_B$ receptor, in screening methods designed to identify compounds which act as receptor ligands and which may be useful to modulate receptor activity. In general terms, such screening methods will involve contacting the receptor protein concerned,

15   preferably the heterodimeric $GABA_B$ receptor, with a test compound and then detecting modulation in the receptor activity, or indeed detecting receptor inactivity, which results. The present invention also includes within its scope those compounds which are identified as possessing useful $GABA_B$ receptor modulation activity, by the screening methods referred to above. The screening

20   methods comprehended by the invention are generally well known to persons skilled in the art, and are further discussed in the experimental section which follows.

Another aspect of the present invention is the use of compounds which have

25   been identified by screening techniques referred to above in the treatment or prophylaxis of disorders which are responsive to modulation of a $GABA_B$ receptor activity, in a mammal. By the term "modulation" what is meant is that there will be either agonism or antagonism at the receptor site which results from ligand binding of the compound at the receptor. $GABA_B$ receptors have been

30   implicated in disorders of the central nervous system (CNS), gastrointestinal (GI) tract, lungs and bladder and therefore modulation of $GABA_B$ receptor activity in these tissues will result in a positive therapeutic outcome in relation to such disorders. In particular, the compounds which will be identified using the screening techniques according to the invention will have utility for treatment

35   and/or prophylaxis of disorders such as spasticity, epilepsy, Alzheimer's

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disease, pain as well as affective disorders and feeding disorders. It is to be understood however, that the mention of such disorders is by way of example only, and is not intended to be limiting on the scope of the invention.

5   The compounds which are identified according to the screening methods outlined above may be formulated with standard pharmaceutically acceptable carriers and/or excipients as is routine in the pharmaceutical art, and as fully described in Remmington's Pharmaceutical Sciences, Mack Publishing Company, Eastern Pennsylvania, 17th Ed, 1985, the disclosure of which is
10   included herein in its entirety by way of reference.

The compounds may be administered via enteral or parenteral routes such as via oral, buccal, anal, pulmonary, intravenous, intraarterial, intramuscular, intraperitoneal, topical or other appropriate administration routes.

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Other aspects of the present invention will be further explained, by way of example, in the appended experimental section.

Experimental

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Results

1. Cloning of Human $GABA_B$-R1 and a novel Receptor subtype, $GABA_B$-R2
Human homologues to the rat $GABA_B$-R1a and 1b splice variants were identified
25   from ESTs and subcloned from Human cerebellum cDNA, using a combination of PCR and Rapid amplification of cDNA ends (RACE) PCR. Human $GABA_B$-R1a and 1b sequences reveal over 99% identity to the rat $GABA_B$-R1a and $GABA_B$-R1b (data not shown). These receptors, like their rat counterparts, both have signal sequences, followed by extended N-termini, a typical seven-
30   transmembrane topology and short intracellular C-terminal tail. The N-terminus encodes the GABA binding domain, which is predicted by limited homology to bacterial periplasmic proteins to exist as two globular domains that capture GABA (Bettler et al., 1998), as well as three potential N-glycosylation sites. Interestingly the $GABA_B$-R1a splice variant N-terminus encodes 129 amino acids
35   over that of $GABA_B$-R1b, which encode two tandem copies of the 'short

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consensus repeat' or sushi domain. Sushi domains are approximately 60 amino acids in length and exist in a wide range of proteins involved in complement and cell-cell adhesion (Chou and Heinrikson, 1997). Therefore the sushi domains within GABA$_B$-R1a may direct protein–protein interactions, possibly through cell-

5   cell contact and may reflect a further role for GABA$_B$-R1a, over and above that of GABA$_B$-R1b. Interestingly during the isolation of these clones, a novel N-terminal splice variant, GABA$_B$-R1c was identified. GABA$_B$-R1c differs from GABA$_B$-R1a by a 185bp deletion from bases 290 to 475 (see Figure 2). This region encodes one of the two Sushi domains unique to GABA$_B$-R1a and therefore the GABA$_B$-

10 R1a and GABA$_B$-R1c splice variants, together with their cellular localisation, may be significant in the biology of GABA$_B$ receptors. Indeed, *in situ* hybridisations suggest that GABA$_B$-R1a and GABA$_B$-R1b have different sub-cellular localisations, with GABA$_B$-R1a expressed at pre-synaptic rather than at post-synaptic sites (Bettler *et al.*, 1998).

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Database searches also identified a number of ESTs showing weaker homology to GABA$_B$-R1, suggesting the existence of a novel GABA$_B$ receptor subtype. Using PCR on Human Brain cerebellum cDNA, we confirmed the existence of such a novel GABA$_B$ receptor which we cloned and sequenced (Figure 1). This

20 novel receptor, which we have called GABA$_B$-R2, shows an overall 54% similarity and 35% identity to GABA$_B$-R1 over the full length of the protein (Figure 2). As expected, hydrophobicity profiles for GABA$_B$-R2 (Figure 3) suggested that the protein has a 42 amino acid signal peptide followed by an extracellular N-terminal domain comparable in size to that of GABA$_B$-R1b and

25 seven membrane spanning regions. In total five N-glycosylation sites were predicted over the N-terminal domain, three of which are conserved within GABA$_B$-R1. Finally, the receptor encodes an intracellular C-terminal domain, which is considerably larger than that of GABA$_B$-R1. No sushi domains were identified within GABA$_B$-R2 sequence and we have no evidence for any splice

30 variants to date.

2. Tissue Distribution

Expression levels of both GABA$_B$-R1 and GABA$_B$-R2 were determined and compared in different tissues and developmental stages by probing Human RNA

35 Master Blots (Clontech). These blots contain polyA$^+$ RNA samples from 50

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human tissues that have been normalized to the mRNA expression levels of eight different "housekeeping" genes. $GABA_B$-R1 levels were examined using a pan-specific probe covering all splice variants (Figure 4a) and the blots indicate that in accordance with the observations of Kaupmann et al., (1997), $GABA_B$-R1 is highly expressed in the CNS, in all areas of the brain and spinal cord. However, in contrast to Kaupmann et al., (1997), we find that $GABA_B$-R1 is also expressed at comparable levels in peripheral tissues, with particularly high levels of expression in the pituitary, lung, ovary, kidney, small intestine, and spleen. In marked contrast, $GABA_B$-R2 is specifically expressed at high levels only in the CNS, with the possible exception of spinal cord where expression appears somewhat lower. No signal is seen for peripheral tissues, in either adult or fetal tissues (Figure 4b). This markedly different distribution of mRNA levels between $GABA_B$-R1 and $GABA_B$-R2 suggests that the two subtypes may have distinct roles in the CNS and periphery.

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3. Initial Expression studies

We reasoned that $GABA_B$-R2 could be a high affinity $GABA_B$ receptor and therefore, expressed the receptor in both Xenopus oocytes and HEK293T cells and looked for functional responses. However, despite repeated attempts, we were unable to detect any functional activation of $GABA_B$-R2 or indeed, $GABA_B$-R1a, $GABA_B$-R1b or $GABA_B$-R1c receptors by either GABA itself or $GABA_B$ selective agonists (See Figures 9, 11 and 12). Several lines of evidence clearly indicated that $GABA_B$-R1 was not expressed as predicted in vivo. Firstly, flow cytometry of HEK293T cells, expressing $GABA_B$-R1b, revealed that receptors were retained on internal membranes rather than expressed at the cell surface (Figure 7). Secondly, $GABA_B$-R1a and $GABA_B$-R1b were expressed as immature glycoproteins, by virtue of their sensitivity to endoglycosidases F and H (Figure 8, lanes 1-6) and finally, $GABA_B$-R1 co-expression in oocytes with either GIRK or CFTR, gave no indication of a functional response (data not shown). We concluded that some additional co-factor must be required to promote a functional response.

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4. Yeast Two Hybrid library screening

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The calcitonin-receptor like receptor is retained as an immature glycoprotein within the endoplasmic reticulum and requires an accessory protein from the recently identified RAMP protein family to transport the receptor to the surface to generate a functional CGRP (Calcitonin gene-related peptide) or adrenomedullin

5   receptor (McLatchie et al., 1998). We anticipated that GABA$_B$-R1 receptors should require an analogous trafficking factor or some other protein co-factor for its transport to the cell surface to generate a high affinity receptor. To identify such potential interacting proteins, a yeast two hybrid library screen was run using the C-terminal 108 amino acids of GABA$_B$-R1 against a Human Brain

10   cDNA library. Interestingly, motif searches revealed a strong coiled-coil domain within these 108 residues, a structure known to mediate protein-protein interactions (Lupas, 1996). From a total of 4.3 x 10^6 cDNAs, 122 positives hits were recovered, 33 of which encoded the whole C-terminal domain of GABA$_B$-R2. This domain of the GABA$_B$-R2 is likewise predicted to contain a coiled-coil

15   motif, which aligns exactly with that of GABA$_B$-R1 (see Figure 2). This observation strongly suggests that the two receptors interact via their C-termini to form a heterodimer. Significantly, the screen did not retrieve the C-terminal domain of the GABA$_B$-R1 itself, implying that GABA$_B$-R1 is unable to homodimerise. This interaction was tested directly in the yeast two hybrid

20   system using the C-termini of the two receptors (Figure 5). GABA$_B$-R1 and GABA$_B$-R2 were able to strongly interact via their C-termini, whilst neither receptor was able to homodimerise. This observation suggested that GABA$_B$-R1 and GABA$_B$-R2 form heterodimers via their C-terminal coiled-coil domains and led to speculation that homodimerisation may bring about a functional binding

25   site in vivo. Therefore, we next confirmed the interaction between the two receptor subtypes by immunoprecipitation studies upon whole epitope-tagged receptor in transfected HEK293T cells.

5. Co-immunoprecipitation Studies.

30   Epitope tagged receptors, Myc-GABA$_B$-R1b and HA-GABA$_B$-R2 were transiently expressed in HEK293T cells either alone or in combination. Immunoprecipitation of Myc-GABA$_B$-R1b from detergent-solubilised cell fractions with Myc antisera led to immunodetection of HA-GABA$_B$-R2 within immune complexes using HA as the primary antibody, but only upon receptor co-expression (Figure 6, lanes 1-3).

35   GABA$_B$-R1 and GABA$_B$-R2 association was confirmed by co-immunodetection of

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Myc-GABA$_B$-R1b from immune complexes captured using the anti-HA antibody. Once again, co-immunoprecipitation could only be seen when the two receptor forms were co-expressed (Figure 6, lanes 4-6). Hence in agreement with the yeast two hybrid observations, these data provide compelling evidence for

5   heterodimerisation between full-length expressed GABA$_B$-R1 and GABA$_B$-R2 in mammalian cells. Therefore, we next examined GABA$_B$ receptor responses following co-expression of both receptor subtypes in HEK293T cells or in *Xenopus* oocytes.

10   6. Surface Expression of the Heterodimer
HEK293T cells were transiently transfected with Myc-GABA$_B$-R1b alone or in combination with HA-GABA$_B$-R2 and transfectants analysed by flow cytometry (Figure 7). Myc-immunoreactivity could not be detected on the surface of cells transfected with Myc-GABA$_B$-R1b alone (Figure7a), although cell

15   permeabilisation revealed immunoreactivity in 35% (n=3) of the cell population (Figure 7b). This latter observation indicated that cells were efficiently transfected and suggested that expressed Myc-GABA$_B$-R1 receptors were localised exclusively on internal membranes. In contrast, 14% (n=3) of HEK293T cells transfected with HA-GABA$_B$-R2 showed surface

20   immunoreactivity (Figure 7c). However, co-transfection of both Myc-GABA$_B$-R1b and HA-GABA$_B$-R2 led to the appearance of Myc-GABA$_B$-R1b on the surface of 20% (n=3) of cells analysed (Figure 7a), strongly suggesting that co-expression of GABA$_B$-R1b with GABA$_B$-R2 is necessary for surface expression of GABA$_B$-R1b.

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7. Receptor Glycosylation studies
Endoglycosidases F and H can be used to differentiate between core and terminally glycosylated *N*-linked glycoproteins. Therefore, these enzymes were used to examine the glycosylation status of both GABA$_B$-R1 and GABA$_B$-R2

30   following expression in HEK293T cells. Membranes from transfected cells were treated with either endoglycosidase F or endoglycosidase H and expressed GABA$_B$ receptors were characterised by immunoblotting to compare relative electrophoretic mobilities of the receptors (Figure 8). Cell membranes expressing either GABA$_B$-R1a or 1b produced distinct bands of M_r 130 and 100K

35   respectively (Figure 8, lanes 1 and 4) which following endoglycosidase F

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treatment, decreased in size to single immunoreactive species of M_r 110 and 80K; representing GABA$_B$-R1a and GABA$_B$-R1b respectively (Figure 8, lanes 2 and 5). This shows that recombinant GABA$_B$-R1a and 1b are glycoproteins, in agreement with the observations of Kaupmann *et al.*, (1997). However, both GABA$_B$-R1a and 1b splice variant forms were also sensitive to endoglycosidase H treatment, indicating that the expressed proteins are only core glycosylated (lanes 3 and 6) and lack terminal glycosylation. This observation, together with the FACS analysis, suggests that the proteins are immaturely glycosylated and retained on internal membranes. Significantly, when either GABA$_B$-R1a (lanes 7-9) or GABA$_B$-R1b (lanes 10-12) was co-expressed with HA-GABA$_B$-R2, a component of GABA$_B$-R1a or 1b was resistant to endoglycosidase H digestion suggesting that when co-expressed with GABA$_B$-R2, a significant fraction of GABA$_B$-R1 is now a mature glycoprotein (lanes 9 and 12).

Similar studies with HA-GABA$_B$-R2 gave an immunoreactive species with an M_r of 120 K (Figure 8, lanes 13, 16, 19) which was sensitive to endoglycosidase F (lanes 14, 17 and 20) but resistant to endoglycosidase H (lanes 15, 18 and 21) treatment, whether expressed alone or in combination with GABA$_B$-R1. Thus, these data indicate that expressed HA-GABA$_B$-R2 is a mature glycoprotein whose glycosylation status is not affected by co-expression with GABA$_B$-R1. Thus, heterodimerisation between GABA$_B$-R1 and GABA$_B$-R2, possibly in the Golgi complex, could be a prerequisite for maturation and transport of GABA$_B$-R1 to the plasma membrane.

8. Functional studies

To determine whether co-expression of GABA$_B$-R1 and GABA$_B$-R2 and its subsequent mature glycosylation and cell surface expression, generated a receptor complex able to functionally respond to GABA, we measured three types of signalling. We used transiently transfected HEK239T cells to examine firstly, activation of [^{35}S]GTPγS binding in membranes and secondly, inhibition of forskolin stimulated cAMP activation in whole cells. Thirdly we expressed GABA$_B$-R1 and GABA$_B$-R2 in *Xenopus* oocytes, expressing either the cystic fibrosis transmembrane regulator (CFTR) or inwardly rectifying K$^+$ channels (GIRK and KATP) and examined activation of ion flux in response to agonist.

i. [^{35}S]GTPγS binding

No GABA stimulated [^{35}S]GTPγS binding was observed in membranes prepared from cells transfected with either GABA$_B$-R1 or HA-GABA$_B$-R2 in combination with G$_{o1}$α. However, co-expression of GABA$_B$-R1 and HA-GABA$_B$-R2 together with G$_{o1}$α resulted in a robust stimulation of [^{35}S]GTPγS binding activity (Figure 9a). This was found to be concentration-dependent with similar EC$_{50}$ (mean, ± S.E.M., n = 3) values determined for membranes from cells transfected with HA-GABA$_B$-R2 and G$_{o1}$α together with either GABA$_B$-R1a (9.5 ± 1.1 x 10^{-5}M) or GABA$_B$-R1b (7.8 ± 0.4 x 10^{-5}M) (Figure 9b). These values are equivalent to those of GABA-mediated stimulation of [^{35}S]GTPγS binding to rat brain membranes (5.9 ± 0.4 x 10^{-5}M) (data not shown). We were concerned that an N-terminal HA epitope tag on GABA$_B$-R2 could alter receptor function and so we performed parallel studies in HEK293T cells, expressing untagged versions of GABA$_B$-R2 and GABA$_B$-R1 together with G$_{o1}$α. Similar efficacies and potencies of GABA action were observed in membranes from these cells, as reported for the epitope tagged receptors (data not shown), clearly suggesting that the addition of these peptide sequences to the N-termini of GABA$_B$-R2 and GABA$_B$-R1 did not significantly alter receptor function. It is noteworthy that a measurable GABA-mediated elevation of [^{35}S]GTPγS binding activity was only observed upon co-expression of GABA$_B$-R1 and HA-GABA$_B$-R2 together with additional G$_{o1}$α (Figure 10). The requirement for additional G protein is most likely due to relatively low levels of endogenously expressed G$_{i/o}$ family G proteins, thus precluding a discernible GABA-mediated response upon GABA$_B$-R1 and GABA$_B$-R2 co-expression.

ii cAMP inhibition

Similar results were obtained from HEK293T cells transiently transfected with GABA$_B$-R1 and GABA$_B$-R2, using inhibition of forskolin evoked cAMP as a readout. Once again, functional responses were only observed when both GABA$_B$-R1 and GABA$_B$-R2 were co-expressed (Figure 11).

iii *Xenopus* oocytes

Xenopus oocytes can assay for three classes of G-protein:

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1) Endogenous oocyte Ca^{2+}-activated chloride conductance can assay for activation of G_q and a subsequent rise in intracellular calcium (Uezono *et al.*,1993).

2) Cystic fibrosis transmembrane regulator (CFTR), which contains a cAMP-activated chloride channel, can assay for receptor activation via G_s or $G_{i/o}$ (Uezono *et al.*,1993; Wotta *et al.*,1997).

3) G-protein regulated potassium channels GIRK1 (Kir 3.1; Kubo *et al.*,1993) and GIRK4 (or CIR, Kir 3.4, Kaprivinsky *et al.*,1995), injected in equal amounts to generate a heteromeric channel, can assay for activation of pertussis toxin sensitive G-proteins (Kovoor *et al.*,1997).

No functional responses to GABA or baclofen were seen when cloned $GABA_B$-R1a, $GABA_B$-R1b or $GABA_B$-R2 receptors were expressed in oocytes in combination with CFTR or GIRK1/4 (data not shown; see Figure 12b). When $GABA_B$-R1 and $GABA_B$-R2 were co-expressed with CFTR, several significant, robust responses were recorded following application of 100μM GABA (Figure 12a). Moreover, repeated application of GABA led to a progressive increase in the size of the CFTR response, suggesting that the functional response of the heterodimer is now sensitised to further challenge by agonist. This phenomenon has not been observed for other cloned receptors expressed in oocytes and may be related to the heterodimerisation or even oligomerisation of the $GABA_B$ receptors. Finally, two other $GABA_B$-selective agonists, Baclofen and SKF97541 elicited similar functional responses through CFTR to that of GABA (Figure12a). In contrast, antagonists gave no response (data not shown).

Next, we examined the $GABA_B$-R1/$GABA_B$-R2 heterodimer with the G-protein regulated potassium channels GIRK1 and GIRK4 and once again found agonist dependant responses. Time course plots were examined for three individual oocytes expressing $GABA_B$-R2 alone (left panel), $GABA_B$-R1 plus $GABA_B$-R2 (middle panel) and the adenosine A1 receptor (as a positive control, right panel) (Figure 12b). In each case, switching from a low potassium physiological solution (ND96) to a high potassium extracellular solution (90mM K^+) led to an inward shift in holding current, resulting from agonist-independent influx of

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potassium ions through the GIRK1/4 channel. No GABA response was seen in oocytes expressing $GABA_B$-R2 in isolation (Figure 12b, left panel) and similarly, $GABA_B$-R1a and $GABA_B$-R1b expressed alone also gave no response to GABA (data not shown). Significantly, a large GABA response was recorded in oocytes co-expressing $GABA_B$-R1 and $GABA_B$-R2 (Figure 12b, middle panel) of a similar magnitude to that of the adenosine A1 receptor in response to the agonist NECA (Figure 12b, right panel). Thus, once again co-expression of the two receptor subtypes elicits a functional agonist-dependant response, whereas expression of either subtype receptor alone does not. We also examined whether co-expression of the two receptors in oocytes could activate endogenous Ca^{2+}-activated chloride conductance. No evidence for activation was seen (data not shown) suggesting that at least in oocytes, the $GABA_B$-R1/$GABA_B$-R2 receptor complex does not signal through G_q. Finally, a current-voltage curve were constructed for an oocyte co-expressing $GABA_B$-R1 and $GABA_B$-R2 (Figure 13). This clearly demonstrates that GABA, bound to the $GABA_B$-receptor, activates a large inwardly rectifying current consistent with activation of the GIRK potassium channel in a fully dose dependant manner.

9. Stoichiometric Studies on the Heterodimer

Since co-expression of $GABA_B$-R1 and $GABA_B$-R2 is necessary for a functional $GABA_B$ receptor, we decided to investigate stoichiometric ratio between the two receptor subtypes *in vivo*. Relative levels of expression for both $GABA_B$-R1 and $GABA_B$-R2 were measured following transfection into HEK293T cells and compared to receptor function, as determined by GTPγS binding (Figure 14). Increasing amounts of HA-$GABA_B$-R1 (up to 1μg) plasmid were transfected into HEK293T cells along with a constant (1μg) amount of HA-$GABA_B$-R2. GABA caused stimulation of [^{35}S]GTPγS binding above basal levels in membranes extracted from these cells, which increased with increasing amount of transfected HA-$GABA_B$-R1 until binding reached a plateau when levels of HA-$GABA_B$-R1 were greater than 0.25μg (Figure 14a). Immunoblotting of the same membrane samples revealed equivalent levels of expression of HA-$GABA_B$-R1 and HA-$GABA_B$-R2 in membranes transfected with 0.25-0.5 μg of HA-$GABA_B$-R1 (Figure 14b). This corresponded to the plateau of GABA-mediated elevation of [^{35}S]GTPγS binding activity and therefore strongly suggests that $GABA_B$-R1 and $GABA_B$-R2 functionally interact in a 1:1 stoichiometric ratio.

10. Competition Binding Studies

Finally, we determined whether the observed functional responses were due to a high affinity $GABA_B$ receptor, composed of a heterodimer of the two receptors. HEK293T cells were transfected with either 1μg $HA-GABA_B-R1b$ and $HA-GABA_B-R2$ individually or with increasing amounts (up to 1μg) of $HA-GABA_B-R1b$ and a fixed amount (1μg) of $HA-GABA_B-R2$ together with $G_{o1}\alpha$. Competition binding assays were then performed upon purified membranes. Expression of $HA-GABA_B-R1b$ alone produced high levels of specific binding of $[^3H]$-CGP54626 (Bittiger et al., 1992), a structural analogue of $[^{125}I]$-CGP64213 and the antagonist originally used to expression clone $GABA_B-R1$ (Kaupmann et al., 1997). However, as previously reported for $[^{125}I]$-CGP64213, GABA inhibition curves were significantly shifted to the right compared with binding to rat brain membranes (Figure 15), giving approximately 22-fold lower IC_{50} than rat brain binding. Significantly, co-expression of equivalent amounts of $HA-GABA_B-R1b$ and $HA-GABA_B-R2$ protein revealed high levels of specific binding. In a control experiment using untagged receptors similar values were obtained (data not shown). Achievement of a 1:1 stoichiometric ratio of expression of $HA-GABA_B-R1b$ and $HA-GABA_B-R2$ led to agonist inhibition curves similar to those obtained in rat brain membranes ($IC_{50} \pm 95\%$ confidence intervals for 1μg $HA-GABA_B-R2$ / 0.25μg $HA-GABA_B-R1b$ = 2.29μM (1.48-3.55μM) and for rat brain = 1.04μM (0.69-1.58μM). Such comparable levels of receptor expression were also shown to permit optimal agonist activation in the GTPγS assay (see Figure 14). Alteration of receptor ratio from 1:1, such that $GABA_B-R1b$ was the most prevalent receptor, led to reduced agonist affinity, presumably due to binding at non-dimerised and immaturely glycosylated $GABA_B-R1b$ receptors (Figure 15). In addition, despite its apparent cell surface expression, we were unable to detect any $[^3H]$-CGP54626 specific binding to HEK293T cells transiently transfected with $HA-GABA_B-R2$ alone (data not shown). We conclude that heterodimerisation of the $GABA_B-R1$ and $GABA_B-R2$ subtypes are necessary to generate a high affinity $GABA_B$ receptor. There are a number of possible explanations for the change in GABA affinity following co-expression of the two receptor subtypes. Appearance of the $GABA_B$ receptor complex at the cell surface would be expected to allow G protein coupling of the receptor which

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would increase agonist affinity. However, in previous studies is has been shown that the lack of G protein coupling alone cannot account for the difference in agonist affinity between rat brain receptors and $GABA_B$-R1 (Kaupmann et al., 1997). Furthermore, we have noted that [^{3}H]-CGP54626 appears to primarily

5   bind the low affinity state of the receptor, even in rat brain membranes, as demonstrated by the fact that GTPγS is unable to shift agonist inhibition curves and actually increases the level of ^{3}H-CGP54626 specific binding (data not shown). Therefore, a more likely explanation for the change in GABA affinity following co-expression of the two $GABA_B$ receptors is that heterodimerisation

10   together with the mature glycosylation state of the protein, produces a binding site conformation with an inherent higher affinity.

Discussion

15   Functional $GABA_B$ receptors within the CNS comprise a cell surface heterodimer of two distinct 7-transmembrane receptor subunits, $GABA_B$-R1 and $GABA_B$-R2 in a 1:1 stoichiometric ratio. In vivo, $GABA_B$ receptors may exist simply as heterodimers or form even larger multimeric complexes of many heterodimers. Formation of the heterodimer via the coiled-coil domains within the receptor C-

20   terminal tails appears to be a pre-requisite for transport and full glycosylation of $GABA_B$-R1, as well as for the generation of a high affinity $GABA_B$ receptor at the cell surface. Using this information, we have been able to reproduce $GABA_B$ sites in both mammalian HEK293T cells as well as in oocytes, using several functional readouts such as activation of ion flux through CFTR or GIRK in

25   oocytes, or inhibition of adenylyl cyclase in HEK293T cells. Indeed the lack of functional responses in cells expressing $GABA_B$-R1 alone and the need for expression of a second 7TM receptor explains why many groups have encountered extreme difficulty in expression cloning a $GABA_B$ receptor via conventional means. We believe this is the first report of receptor

30   heterodimerisation as an obligate requirement to generate a high affinity, fully functional receptor in recombinant systems, which is fully equivalent to that of endogenous tissues.

Dimerisation has been reported for other receptor families, such as the opioid

35   family as a part of their desensitisation process, the β2-adrenergic receptor,

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where homodimers may play a role in signalling, and the metabotropic glutamate receptors (mGluRs, Hebert et al., 1996; Romano et al., 1996; Cvejic et al., 1997, Hebert and Bouvier, 1998). Significantly, dimerisation in these receptor families does not appear to be an absolute requirement for functional coupling in

5 recombinant systems. In the case of the mGluRs, which are a closely related receptor family to GABA_B (Kaupmann et al., 1997), homodimerisation is mediated through disulphide bridges between the N-terminal extracellular domains rather than a C-terminal coiled-coil. Indeed, heterodimerisation between two 7-transmembrane receptors, leading to both trafficking and mature

10 glycosylation of the proteins to yield a functional receptor is unprecedented and is unique in the GPCR field. Certainly, mGluRs have not been found to form heterodimers (Romano et al., 1996) and the fact that two such closely related receptors families have evolved such different mechanisms of dimer formation suggests that this is a fundamentally important process for receptor function.

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In vivo, pharmacological evidence suggests that there are many different GABA_B receptor subtypes, both within the CNS as well as in peripheral tissues. How are such pharmacological subtypes of GABA_B receptors formed? Only GABA_B-R1 and GABA_B-R2 have been identified as separate genes to date and database

20 trawling has not identified any further receptors homologous to known GABA_B receptors. This does not exclude the possibility that more, as yet unrecognised GABA_B receptors do exist. Differences in distribution exist for the two GABA_B receptors, for example GABA_B-R2 is specifically expressed in the CNS whereas GABA_B-R1 is expressed in both central and peripheral sites. These differences

25 in distribution clearly add further complexity leading to the pharmacologically distinct receptor subtypes. Moreover, the genes encoding the GABA_B receptors may be differentially spliced. GABA_B-R1 encodes three N-terminal splice variants and yet more may remain to be detected. Interestingly, these splice variants have alterations in their N-terminal extracellular domain, the region

30 involved in GABA binding (Takahashi et al., 1993, O'Hara et al., 1993) and encode either two (GABA_B-R1a), one (GABA_B-R1c) or no (GABA_B-R1b) sushi domains. Given that the sushi domains mediate cell-cell protein-protein contact, the differences in these three splice variants may account for yet more of the pharmacologically defined GABA_B receptor subtypes. To date, we have not

35 detected any splice variants to GABA_B-R2. Furthermore there are significant

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differences in the distribution of the individual splice variants suggesting that they may serve different functions within the CNS. For instance, $GABA_B$-R1a splice variant is reported as presynaptic within the brain (Bettler et al., 1998) and therefore may define presynaptic $GABA_B$ autoreceptors. It seems likely that

5   these splice variants of $GABA_B$-R1 may account for at least some of the pharmacologically defined subtypes. Finally, with this novel observation of obligate receptor heterodimerisation, a further level of complexity has been added since functional $GABA_B$ binding sites require a heterodimerisation partner.

10   Now the molecular nature of the $GABA_B$ receptor is more fully understood, recombinant systems can be established for high throughput screening for compounds against individual pharmacologically defined $GABA_B$ sites. By these means, compounds with greater specificity and with fewer unwanted side effects can be discovered. For this, $GABA_B$-R1 and $GABA_B$-R2 (including all spice

15   variants, and any fragments of the receptor) should be co-expressed either stably or transiently in suitable host cells. Suitable host cells include higher eukaryotic cell lines, such as mammalian cells, insect cells, lower eukaryotic cells, such as yeast or prokaryotic cells such as a bacterial cells. Screening assays with these recombinant cell lines could involve the use of radioligand

20   binding to the dimer or individual subunits within the dimer. The activity profile in a binding assay to the dimer is likely to be different from the activity of compounds assayed using binding assays to $GABA_B$-R1 alone due to alterations in the glycosylation status and the conformation of the receptor as a result of co-expressing $GABA_B$-R1 or $GABA_B$-R2. Functional assays, which measure events

25   downstream of receptor activation, can also be used for screening compounds. Such assays include [^{35}S]-GTPγS binding to membranes isolated from cells expressing the dimer; activation or inhibition of ion channels using electrophysiological recording or ion flux assays; mobilisation of intracellular calcium; modulation of cAMP levels; activation or inhibition of MAP kinase

30   pathways or alterations in the activity of transcription factors with the use of reporter genes. Further to this, secondary screens can be established in a similar manner, using different heterodimer combinations to exclude unwanted activity and thereby establish subtype selective $GABA_B$ compounds.

28

In addition, any approach targetting the disruption or enhancemant of dimer formation of the GABA$_B$ heterodimer could represent a novel therapeutic approach with which to target GABA$_B$ receptors. Such strategies could include peptides or proteins physically associated with the coiled-coil domain or indeed,

5   any other interacting regions of the dimer. Small molecules could also be identified which act at the points of contact formed by interaction of the components of the dimer. These may either promote or enhance the receptor function. Finally, antibodies could be made which specifically recognise epitopes on the dimer, as opposed to the monomer subunits. These could be used as

10   tools to further elucidate the function of GABA$_B$ receptors in disease or as therapeutic agents in their own right.

Methods
DNA Manipulation

15   Standard molecular biology protocols were used throughout (Sambrook *et al.,* 1989) and all bacterial manipulations used Escherichia coli XL-1Blue (Stratagene) according to the manufacturers instructions. Standard PCR conditions were used throughout, unless otherwise stated. PCR reaction mixture contained 10-50ng of target DNA, 1 pmol of each primer; 200μM dNTPs and

20   2.5U of either Taq polymerase (Perkin-Elmer) or Pful polymerase (Stratagene) with the appropriate buffer as supplied by the manufacturer. Cycling parameters were 1cycle 95°C 2mins; 25 cycles 95°C 45secs 55°C 45secs 72°C 1min; 1cycle 72°C 10mins. All PCR were carried out using either a Perkin Elmer 9600 PCR machine or a Robocycler Gradient 96 (Stratagene) PCR machine.

25

GABA$_B$-R1 – Cloning of Human homologues and splice variants
Several human EST's (X90542; X90543; D80024; AA348199; T06711; T07518 and AA38224) were identified as homologous to the rat GABA$_B$-R1a and GABA$_B$-R1b sequences (Y10369; Y10370). The ESTs were aligned and the

30   predicted open reading frame was amplified by RT-PCR from human brain cerebellum polyA$^+$ RNA (Clontech) using the Superscript Preamplification System (Life Technologies). The 3' end of the receptor (1545-2538bp; GABA$_B$-R1b) was amplified using primers 5'-GCGACTGCTGTGGGCTGCTTACT GGC-3 and 5'-GCGAATTCCCTGTCCTCCCTCACCCTACCC-3'. The central

35   section (277–1737bp of GABA$_B$-R1b) was amplified using 5'-

29

CCGAGCTCAAGCTCATCCACCACG-3'   and   5'-
TCTTCCTCCACTCCTTCTTTTCTT-3'. PCR products were subcloned into pCR-
Script SK(+) (PCR-script Amp cloning kit; Stratagene). Error free PCR product
were assembled in a three-way BstEII, SacI and EcoRI ligation and subcloned

5   into pBluescript SK (-) (Stratagene).

The N-termini of the splice variants were generated using RACE (rapid
amplification of cDNA ends) PCR with the Marathon cDNA amplification kit
against Marathon-Ready human cerebellum cDNA (Clontech). RACE PCR was

10   primed from a conserved sequence within $GABA_B$-R1 using primer 5'-
TGAGCTGGAGCCATAGGAAAGCACAAT-3' to generate a 700bp product. This
further PCR amplified using the AP2 primer (Marathon) and a second internal
$GABA_B$-R1 primer 5'-GATCTTGATAGGGTCGTTGTAGAGCA-3'. The resulting
600bp product was subcloned using the Zero blunt PCR cloning kit (Invitrogen).

15   Sequence information achieved from this RACE PCR was used to clone the N-
terminus of the $GABA_B$-R1b splice variant, using primers 5'-
GCTCCTAACGCTCCCCAACA-3' and 5'-GGCCTGGATCACACTTGCTG-3' into
pCR-Script SK (+)(Stratagene). Human $GABA_B$-R1a 5' sequences were
retrieved from Incyte database EST's (1005101;3289832) and used to design

20   primers   5'-CCCAACGCCACCTCAGAAG-3'   and   5'-
CCGCTCATGGGAAACAGTG C-3'. PCR on cerebellum cDNA and KELLY
neuroblastoma cell line cDNA produced two discreet bands at 300bp and 400bp,
which were cloned into pCR-Script SK (+) (Stratagene). Sequencing revealed
that the 400bp product encoded some of the Human $GABA_B$R1a 5' sequences

25   and the 300bp product encoded the novel splice variant, $GABA_B$-R1c. Next,
primer, 5'-CCCCGGCACACATACTCAATCTCATAG-3' was designed to RACE
PCR the missing ~225bp of $GABA_B$-R1a. A 250bp product was obtained and
reamplified using primer 5'-CCGGTACCTGATGCCCCCTTCC-3' with primer
AP2 (Marathon). A ~250bp band was once again generated, subcloned into

30   pCR-Script SK (+) and when sequenced, encoded the 5' end of $GABA_B$-R1a.
Next, clones spanning both the conserved receptor sequence and the %' ends
of the splice variants $GABA_B$-R1a and $GABA_B$-R1c were generated. Primer 5'-
CGAGATGTTGCTGCTGCTGCTA-3', priming from the start codon and the
reverse RACE primer generated a predicted ~800bp band and this was

35   subcloned into pCR-Script SK(+). Now, full-length $GABA_B$-R1a, $GABA_B$-R1b and

30

GABA$_B$-R1c clones can be assembled in pcDNA3.1(-) (Invitrogen). For GABA$_B$-R1b, 5' sequences, restricted NotI /SacI, and the conserved region of the receptor , cut EcoRI /SacI were both co-ligated into pcDNA3.1(-), restricted NotI /EcoRI. Likewise, the GABA$_B$-R1a and GABA$_B$-R1c 5' fragments were subcloned Xhol /SacI with the EcoRI/SacI conserved fragment and co-ligated into pcDNA3.1(-) , cut XhoI /EcoRI to reconstitute full length clones.

Tagging of GABA$_B$-R1b

GABA$_B$-R1b was tagged with either myc or HA epitopes. PCR primers 5'-TAGGATCCCACTCCCCCCATCCC-3' and 5'-CCAGCGTGGAGACAGAGCTG-3' were used to amplify a region immediately following the proposed signal sequence (position 88) to approx. 20bp downstream of a unique PstI site at position 389 of the coding sequence, creating a unique 5' in-frame BamHI site. This fragment was cloned ,BamHI/PstI , into a vector containing the CD97 signal sequence, the myc epitope and an in-frame BamHI site. This construct also contains a NotI site 5' to the CD97 signal sequence and an EcoRI site downstream of the PstI site. GABA$_B$-R1b sequences downstream to the PstI site and upto an external EcoRI site were subcloned from full length receptor into the vector described above likewise cut with PstI/EcoRI, to assemble full length tagged GABA$_B$-R1b. CD97 signal sequence, myc epitope and GABA$_B$-R1b coding sequence were subcloned, NotI/EcoRI, into pCDNA3.1(-) (Invitrogen).

HA epitope was added to GABA$_B$-R1b by co-ligation of the 5' BamHI/PstI and 3' PstI/EcoRI fragments into pCIN6 cut with BamHI/EcoRI. This vector contains a T8 signal sequence and 12CA5 HA epitope immediately preceding an in-frame BamHI site.

Cloning of GABA$_B$-R2, the novel GABA$_B$ receptor subtype

EST clones (H14151, R76089, R80651, AA324303, T07621, Z43654) were identified with approximately 50% nucleotide identity to GABA$_B$-R1. PCR revealed that H14151 contained a 1.5Kb insert and encoded sufficient sequence for a substantial portion the novel GABA$_B$ receptor. PCR between the 3' end of H14151 and the 5' end of AA324303, using a cerebellum cDNA library as template, produced a ~700bp product, which when cloned into the T-vector (TA cloning kit, Invitrogen) and sequenced, revealed that T07621 overlaps within AA324303. Also, Z43654 as well as genomic DNA fragments R76089 and

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R80651 were found to overlap AA324303 and together provided sequence data for the 3' end of the GABA$_B$ subtype receptor. Further sequencing of H14151 provided the full sequence for the novel receptor subtype. However, because of ambiguities in the position of the stop codon in Z43654/R80448/R80651, Incyte

5   clones 662098 and 090041, which overlap this region, were sequenced. The stop codon was identified and sequence for GABA$_B$-R2 was confirmed as within H14151 (5' end) and 662098 (3' end). 5' sequences of GABA$_B$-R2 were PCR generated using primers 5'-ATGGCTTCCCCGCGGAG-3' to provide the start codon of the receptor and primer 5'-GAACAGGCGTGGTTGCAG-3', priming

10   beyond a unique EagI site. The expected ~250bp product was cloned into pCRSCRIPT and sequenced. Full length receptor was then assembled with a three way ligation between H14151, cut with ApaLI /EagI; 662098, cut with ApaLI /NotI and pCRSCRIPT- GABA$_B$-R2-5' PCR product, restricted by EagI.Full length GABA$_B$-R2 was removed from the pCRSCRIPT vector using EcoRI /NotI

15   and ligated into pcDNA3 (Invitrogen) for expression studies.

HA-epitope tagged GABA$_B$-R2 was constructed in pCIN6,.A linker was constructed encoding amino acids between the GABA$_B$-R2 signal sequence and the unique EagI site.

20

       HindIII XhoI   EagI   EcoRI
AGCTT CTC GAG GCT TGG GGA TGG GCA CGA GGA GCT CCT GCT **CGG CCG G**
           A GAG CTC CGA ACC CCT ACC CGT GCT CCT CGT GGT CGA **GCC GGC CTT AA**
             Ala Trp Gly Trp Ala Arg Gly Ala Pro Arg

25   The linker was cloned into pUC18 (EcoRI /HindIII) followed by full length GABAB-R2, from pCRSCRIPT as an EagI/NotI fragment. Finally, the modified GABA$_B$-R2 was cloned into pCIN6 as a XhoI fragment.

Distribution Studies

30   Blots were hybridized overnight at 65°C according to the manufacturers' instructions with radioactively randomly primed cDNA probes using ExpressHyb Hybridization solution. Probe for GABA$_B$-R1, corresponding to residues 1129-1618 of the GABA$_B$-R1b coding sequence was PCR amplified using primers 5'-CGCCTGGAGGACTTCAACTACAA-3'   and   5'-

35   TCCTCCCAATGTGGTAACCATCG-3' against GABA$_B$-R1b DNA as template.

33

The GAL4$_{BD}$-GABA$_B$-R1 C-terminus fusion in pYTH9 was stably integrated into the trp1 locus of Y190 by targetted homologous recombination. Yeast expressing GAL4$_{BD}$-GABA$_B$-R1 C-terminus were selected and transformed with Human brain cDNA library under leucine selection, using a high efficiency
5   Lithium acetate transformation protocol (Clontech Laboratories, 1998). Sufficient independent cDNAs were transformed to give a three fold representation of the library. Interacting clones were selected by growth under 20mM 3-amino-1,2,4-triazole (Sigma) selection, followed by production of β-galactosidase, as determined by a freeze-fracture assay (Clontech Laboratories, 1998). Plasmid
10   DNA was recovered from yeast cells following digestion of the cell wall by 400µg/ml Zymolase 100T (ICN Biochemicals) in 250µl 1.2M Sorbitol; 0.1M potassium phosphate buffer (pH 7.4) at 37°for 2 h. Plasmid DNA was extracted by standard Qiagen alkaline lysis miniprep as per manufacturers' instructions and transformed into Ultracompetent XL-2Blue cells (Stratagene). Plasmid DNA
15   was sequenced using primer 5'-CAGGGATGTTTAATACCACTACAATGG-3' using automated ABI sequencing and resulting sequences were blasted against the databases.

Yeast Y190 was transformed with pYTH16 and pACT2 expressing GABA$_B$-R1 C-
20   terminal domain and the GABA$_B$-R2 C-terminal domain in all combinations, as well as against empty vectors. Transformants were grown in liquid media to mid-logarithmic phase and approximately 1.5ml harvested. β-galactosidase activity was quantified using substrate o-nitrophenyl β-D-galactopyranoside (ONPG; Sigma) using a liquid nitrogen freeze fracture regime essentially as described by
25   Harshman et al., (1988).

Two-microelectrode voltage-clamp in *Xenopus* oocytes
Adult female *Xenopus laevis* (Blades Biologicals) were anaesthetised using
30   0.2% tricaine (3-aminobenzoic acid ethyl ester), killed and the ovaries rapidly removed. Oocytes were de-folliculated by collagenase digestion (Sigma type I, 1.5 mg ml^{-1}) in divalent cation-free OR2 solution (82.5mM NaCl, 2.5mM KCl, 1.2mM NaH$_2$PO$_4$, 5mM HEPES; pH 7.5 at 25°C). Single stage V and VI oocytes were transferred to ND96 solution (96mM NaCl, 2mM KCl, 1mM MgCl$_2$, 1.8mM

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$CaCl_2$, 5mM HEPES; pH 7.5 at 25°C) which contained 50µg ml^{-1} gentamycin and stored at 18°C.

GABA$_B$-R1a, GABA$_B$-R1b (both in pcDNA3.1rev, Invitrogen), GABA$_B$-R2, GIRK1,
5   GIRK4 (in pcDNA3) and cystic fibrosis transmembrane regulator (CFTR; in pBluescript, Stratagene) were linearised and transcribed to RNA using T7 or T3 polymerase (Promega Wizard kit). m'G(5')pp(5')GTP capped cRNA was injected into oocytes (2? ~9nl of 1µgµl^{-1} RNA per oocyte) and whole-cell currents were recorded usin₂ /o-microelectrode voltage-clamp (Geneclamp amplifier, Axon
10   instruments Inc.) 3 to 7 days post-RNA injection. Microelectrodes had a resistance of 0.5 to 2MΩ when filled with 3M KCl. In all experiments oocytes were voltage-clamped at a holding potential of −60mV in ND96 solution (superfused at 2ml per min.) and agonists were applied by addition to this extracellular solution. In GIRK experiments the extracellular solution was
15   changed to a high potassium solution prior to agonist application, to facilitate the recording of inward potassium currents. Current-voltage curves were constructed by applying 200ms voltage-clamp pulses from the holding potential of −60mV to test potentials between −100mV and +50mV.

20   Mammalian Cell culture and transfections
HEK293T cells (HEK293 cells stably expressing the SV40 large T-antigen) were maintained in DMEM containing 10 % (v/v) foetal calf serum and 2 mM glutamine. Cells were seeded in 60 mm culture dishes and grown to 60-80 % confluency (18-24 h) prior to transfection with pCDNA3 containing the relevant
25   DNA species using Lipofectamine reagent. For transfection, 3 µg of DNA was mixed with 10 µl of Lipofectamine in 0.2 ml of Opti-MEM (Life Technologies Inc.) and was incubated at room temperature for 30 min prior to the addition of 1.6 ml of Opti-MEM. Cells were exposed to the Lipofectamine/DNA mixture for 5 h and 2 ml of 20 % (v/v) newborn calf serum in DMEM was then added. Cells were
30   harvested 48-72 h after transfection.

Preparation of membranes
Plasma membrane-containing P2 particulate fractions were prepared from cell pastes frozen at −80°C after harvest. All procedures were carried out at 4°C.
35   Cell pellets were resuspended in 1 ml of 10 mM Tris-HCl and 0.1 mM EDTA, pH

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7.5 (buffer A) and by homogenisation for 20 s with a polytron homogeniser followed by passage (5 times) through a 25-guage needle. Cell lysates were centrifuged at 1,000 g for 10 min in a microcentrifuge to pellet the nuclei and unbroken cells and P2 particulate fractions were recovered by

5   microcentrifugation at 16,000 g for 30 min. P2 particulate fractions were resuspended in buffer A and stored at –80°C until required. Protein concentrations were determined using the bicinchoninic acid (BCA) procedure (Smith *et al.*, 1985) using BSA as a standard.

10   High affinity [^{35}S]GTPγS binding
Assays were performed in 96-well format using a method modified from Wieland and Jakobs, 1994. Membranes (10 mg per point) were diluted to 0.083 mg/ml in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl$_2$, pH7.4) supplemented with saponin (10 mg/l) and pre–incubated with 40 mM GDP.

15   Various concentrations of GABA were added, followed by [^{35}S]GTPgS (1170 Ci/mmol, Amersham) at 0.3 nM (total vol. of 100 ml) and binding was allowed to proceed at room temperature for 30 min. Non-specific binding was determined by the inclusion of 0.6 mM GTP. Wheatgerm agglutinin SPA beads (Amersham) (0.5 mg) in 25 ml assay buffer were added and the whole was incubated at room

20   temperature for 30 min with agitation. Plates were centrifuged at 1500 g for 5 min and bound [^{35}S]GTPgS was determined by scintillation counting on a Wallac 1450 microbeta Trilux scintillation counter.

Measurement of cAMP levels
25   24 hours following transfection, each 60 mm dish of HEK293T cells was split into 36 wells of a 96-well plate and the cells were allowed to reattach overnight. Cells were washed with PBS and pre-incubated in DMEM medium containing 300 μM IBMX for 30 minutes at 37°C. Forskolin (50 μM) and varying concentrations of GABA were added and cells incubated for a further 30 min prior to cAMP

30   extraction with 0.1M HCl for 1h at 4°C. Assays were neutralised with 0.1 M KHCO$_3$ and cAMP levels determined using scintillation proximity assays (Biotrak Kit, Amersham).

Flow Cytometric Analysis

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HEK293T cells were transiently transfected with cDNA as described. 48-72h following transfection, cells were recovered and washed twice in PBS supplemented with 0.1 % (w/v) NaN_3 and 2.5 % (v/v) foetal calf serum. Cells were resuspended in buffer and incubated with primary antibodies 9E10 (c-Myc)
5   or 12CA5 (HA) for 15 min at room temperature. Following three further washes with PBS, cells were incubated with secondary antibody (sheep anti-mouse Fab_2 coupled with fluorescein isothiocyanate (FITC)) diluted 1:30 for 15 min at room temperature. For permeabilised cells, a Fix and Perm kit (Caltag) was used. Cell analysis was performed on a Coulter Elite flow-cytometer set up to detect
10   FITC fluoresence. 30,000 cells were analysed for each sample.

Immunological studies
Antiserum 501 was raised against a synthetic peptide corresponding to the C-terminal 15 amino acids of the $GABA_B$-R1 receptor and was produced in a
15   sheep, using a conjugate of this peptide and keyhole limpet hemocyanin (Calbiochem) as antigen. Membrane samples 30-60 µg) were resolved by SDS-PAGE using 10 % (w/v) acrylamide. Following electrophoresis, proteins were subsequently transferred to nitrocellulose (Hybond ECL, Amersham), probed with antiserum 501 at 1:1000 dilution and visualised by enhanced
20   chemiluminescence (ECL, Amersham). Epitope tags were visualised by immunoblotting with anti-Myc (9E10; 1:100 dilution) or anti-HA (12CA5; 1:500) monoclonal antibodies.

Deglycosylation
25   Enzymatic removal of asparagine-linked (N-linked) carbohydrate moieties with endoglycosidases F and H was performed essentially according to manufacturers' instructions (Boehringer Mannheim) using 50 µg of membrane protein per enzyme reaction. $GABA_B$ receptor glycosylation status was studied following SDS-PAGE/immunoblotting of samples.
30

Immunoprecipitation procedures
Transiently transfected HEK293T cells were harvested as described above from 60mm culture dishes. Cells from each dish were resuspended in 1 ml of 50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Nonidet® P40, 0.5 % (w/v) sodium
35   deoxycholate, pH 7.5 (lysis buffer) supplemented with Complete™ protease

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inhibitor cocktail tablets (1 tablet/25 ml) (Boehringer Mannheim). Cell lysis and membrane protein solubilisation was achieved by homogenisation for 20 seconds with a polytron homogeniser, followed by gentle mixing for 30 min at 4°C. Insoluble debris was removed by microcentrifugation at 16,000 g for 15 min

5 at 4°C and the supernatant was pre-cleared by incubating with 50 µl of Protein A-agarose (Boehringer Mannheim) for 3 h at 4°C on a helical wheel to reduce non-specific background. Solubilised supernatant was divided into 2 x 500 µl aliquots and 20 µl of either HA or Myc antisera was added to each. Immunoprecipitation was allowed to proceed for 1 h at 4°C on a helical wheel

10 prior to the addition of 50 µl of Protein A-agarose suspension. Capture of immune complexes was progressed overnight at 4°C on a helical wheel. Complexes were collected by microcentrifugation 12,000g for 1 min at 4°C and supernatant was discarded. Beads were washed by gentle resuspension and agitation sequentially in 1 ml of 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1 %

15 (v/v) Nonidet® P40 and 0.05 % (w/v) sodium deoxycholate followed by 1 ml of 50 mM Tris-HCl, pH 7.5, 0.1 % (v/v) Nonidet® P40 and 0.05 % (w/v) sodium deoxycholate. Immunoprecipitated proteins were released from Protein A-agarose by incubation in 30 µl of SDS-PAGE sample buffer at 70°C for 10 min and analysed by SDS-PAGE followed by immunoblotting.

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Binding Assays

Competition binding assays were performed in 50mM Tris HCl buffer (pH7.4) containing 40µM isoguvacine (Tocris Cookson) to block rat brain GABA$_A$ binding sites. P2 membrane preparations were made from HEK293T cells transfected

25 using conditions described above. Increasing concentrations of GABA were added to displace the antagonist [3H]-CGP 54626 (Tocris Cookson, 40Ci/mmol). Assay conditions were 0.4-0.6nM [^{3}H]-CGP54626, incubated with 50µg/tube crude rat brain 'mitochondrial' fractions or 25µg/tube HEK293T P2 membranes at room temperature for 20 minutes. The total volume per tube was 0.5ml and

30 non specific binding was determined using 1mM GABA. Bound ligand was recovered using a Brandel 48 well harvester onto GF/B filters (Whatman) and measured by liquid scintillation using a Beckman LS6500 counter.

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Claims

1. An isolated GABA$_B$-R2 receptor protein or a variant thereof.

2. An isolated GABA$_B$-R2 receptor protein having amino acid sequence provided in Fig. 1B, or a variant thereof.

3. A nucleotide sequence encoding a GABA$_B$-R2 receptor or a variant thereof, or a nucleotide sequence which is complementary thereto.

4. A nucleotide sequence encoding a GABA$_B$-R2 receptor, as shown in Fig. 1A, or a variant thereof, or a nucleotide sequence which is complementary thereto.

5. The nucleotide sequence of either claim 3 or claim 4, which is a cDNA sequence.

6. An expression vector comprising a nucleotide sequence according to any one of claims 3 to 5, which is capable of expressing a GABA$_B$-R2 receptor protein or a variant thereof.

7. A stable cell line comprising a vector according to claim 6.

8. The cell line according to claim 7 which is a modified HEK293T cell line.

9. An antibody specific for a protein as claimed in either claim 1 or claim 2.

10. An isolated GABA$_B$-R1c receptor protein or a variant thereof.

11. An isolated GABA$_B$-R1c receptor protein having amino acid sequence provided in Fig. 2, or a variant thereof.

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12. A nucleotide sequence encoding a $GABA_B$-R1c receptor protein or a variant thereof, or a nucleotide sequence which is complementary thereto.

13. A nucleotide sequence encoding a $GABA_B$-R1c receptor protein or a variant thereof as claimed in claim 11, or a nucleotide sequence which is complementary thereto.

14. A nucleotide sequence of either claim 12 or claim 13 which is a cDNA sequence.

15. An expression vector comprising a nucleotide sequence according to any one of claims 12 to 14, which is capable of expressing a $GABA_B$-R1c receptor protein or a variant thereof.

16. A stable cell line comprising a vector according to claim 15.

17. The cell line according to claim 16 which is a modified HEK293T cell line.

18. An antibody specific for a protein as claimed in either claim 10 or claim 11.

19. A $GABA_B$ receptor comprising an heterodimer between a $GABA_B$-R1 receptor protein or a variant thereof and a $GABA_B$-R2 receptor protein or a variant thereof.

20. The $GABA_B$ receptor according to claim 19 wherein the $GABA_B$-R1 receptor is a $GABA_B$-R1a receptor or variant thereof.

21. The $GABA_B$ receptor according to claim 19 wherein the $GABA_B$-R1 receptor is a $GABA_B$-R1b receptor or variant thereof.

22. The $GABA_B$ receptor according to claim 19 wherein the $GABA_B$-R1 receptor is a $GABA_B$-R1c receptor or variant thereof.

23.   An expression vector comprising a nucleotide sequence encoding for a $GABA_B$-R1 receptor or a variant thereof and a nucleotide sequence encoding for a $GABA_B$-R2 receptor or variant thereof, said vector being capable of expressing both a $GABA_B$-R1 and a $GABA_B$-R2 receptor proteins or variants thereof.

24.   The vector according to claim 23 wherein the $GABA_B$-R1 receptor is a $GABA_B$-R1a receptor or variant thereof.

25.   The vector according to claim 23 wherein the $GABA_B$-R1 receptor is a $GABA_B$-R1b receptor or variant thereof.

26.   The vector according to claim 23 wherein the $GABA_B$-R1 receptor is a $GABA_B$-R1c receptor or variant thereof.

27.   A stable cell line comprising a vector according to any one of claims 23 to 26.

28.   A stable cell line modified to express both $GABA_B$-R1 and $GABA_B$-R2 receptor proteins or variants thereof.

29.   The stable cell line according to either claims 27 or 28 which is a modified HEK293T cell line.

30.   A $GABA_B$ receptor produced by a stable cell line according to any one of claims 27 to 29.

31.   An antibody specific for a receptor as claimed in any one of claims 19 to 22.

32.   A method for identification of a compound which exhibits $GABA_B$ receptor modulating activity, comprising contacting a $GABA_B$ receptor according to any'one of claims 19 to 22 or 30 with a test compound and detecting modulating activity or inactivity.

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33.   A compound which modulates GABA$_B$ receptor activity, identifiable by a method according to claim 32.

34.   A method of treatment or prophylaxis of a disorder which is responsive to modulation of GABA$_B$ receptor activity in a mammal, which comprises administering to said mammal an effective amount of a compound identifiable by the method according to claim 32.

35.   The method according to claim 34 wherein the disorder is a CNS disorder, a GI disorder, a lung disorder or a bladder disorder.

36.   The method according to claim 34 wherein the disorder is spasticity, epilepsy, Alzheimer's disease, pain or an affective or feeding disorder.

37.   Use of a compound identifiable by the method according to claim 32 in a method of formulating a medicament for treatment or prophylaxis of a disorder which is responsive to modulation of GABA$_B$ receptor activity in a mammal.

38.   The use according to claim 36 wherein the disorder is a CNS disorder, a GI disorder, a lung disorder or a bladder disorder.

39.   The use according to claim 36 wherein the disorder is spasticity, Alzheimer's disease, pain or an affective or feeding disorder.

40.   A method of producing a GABA$_B$ receptor comprising introducing into an appropriate cell line a suitable vector or vectors comprising nucleotide sequences encoding for GABA$_B$-R1 and GABA$_B$-R2 receptors or variants thereof, under conditions suitable for obtaining expression of the receptors or variants.

FIG. 1

Nucleotide and protein sequences of Human GABA_B-R2

A. Nucleotide Sequence

```
ATGGCTTCCCCGCGGAGCTCCGGGCAGCCCGGGCCGCCGCCGCCGCCGCCACCGCCGCCC        60
GCGCGCCTGCTACTGCTACTGCTGCTGCCGCTGCTGCTGCCTCTGGCGCCCGGGGCCTGG       120
GGCTGGGCGCGGGGCGCCCCCCGGCCGCCGCCCAGCAGCCCGCCGCTCTCCATCATGGGC       180
CTCATGCCGCTCACCAAGGAGGTGGCCAAGGGCAGCATCGGGCGCGGTGTGCTCCCCGCC       240
GTGGAACTGGCCATCGAGCAGATCCGCAACGAGTCACTCCTGCGCCCCTACTTCCTCGAC       300
CTGCGGCTCTATGACACGGAGTGCGACAACGCAAAAGGGTTGAAAGCCTTCTACGATGCA       360
ATAAAATACGGGCCTAACCACTTGATGGTGTTTGGAGGCGTCTGTCCATCCGTCACATCC       420
ATCATTGCAGAGTCCCTCCAAGGCTGGAATCTGGTGCAGCTTTCTTTTGCTGCAACCACG       480
CCTGTTCTAGCCGATAAGAAAAAATACCCTTATTTCTTTCGGACCGTCCCATCAGACAAT       540
GCGGTGAATCCAGCCATTCTGAAGTTGCTCAAGCACTACCAGTGGAAGCGCGTGGGCACG       600
CTGACGCAAGACGTTCAGAGGTTCTCTGAGGTGCGGAATGACCTGACTGGAGTTCTGTAT       660
GGCGAGGACATTGAGATTTCAGACACCGAGAGCTTCTCCAACGATCCCTGTACCAGTGTC       720
AAAAAGCTGAAGGGGAATGATGTGCGGATCATCCTTGGCCAGTTTGACCAGAATATGGCA       780
GCAAAAGTGTTCTGTTGTGCATACGAGGAGAACATGTATGGTAGTAAATATCAGTGGATC       840
ATTCCGGGCTGGTACGAGCCTTCTTGGTGGGAGCAGGTGCACACGGAAGCCAACTCATCC       900
CGCTGCCTCCGGAAGAATCTGCTTGCTGCCATGGAGGGCTACATTGGCGTGGATTTCGAG       960
CCCCTGAGCTCCAAGCAGATCAAGACCATCTCAGGAAAGACTCCACAGCAGTATGAGAGA      1020
GAGTACAACAACAAGCGGTCAGGCGTGGGGCCCAGCAAGTTCCACGGGTACGCCTACGAT      1080
GGCATCTGGGTCATCGCCAAGACACTGCAGAGGGCCATGGAGACACTGCATGCCAGCAGC      1140
CGGCACCAGCGGATCCAGGACTTCAACTACACGGACCACACGCTGGGCAGGATCATCCTC      1200
AATGCCATGAACGAGACCAACTTCTTCGGGGTCACGGGTCAAGTTGTATTCCGGAATGGG      1260
GAGAGAATGGGGACCATTAAATTTACTCAATTTCAAGACAGCAGGGAGGTGAAGGTGGGA      1320
GAGTACAACGCTGTGGCCGACACACTGGAGATCATCAATGACACCATCAGGTTCCAAGGA      1380
TCCGAACCACCAAAAGACAAGACCATCATCCTGGAGCAGCTGCGGAAGATCTCCCTACCT      1440
CTCTACAGCATCCTCTCTGCCCTCACCATCCTCGGGATGATCATGGCCAGTGCTTTTCTC      1500
TTCTTCAACATCAAGAACCGGAATCAGAAGCTCATAAAGATGTCGAGTCCATACATGAAC      1560
AACCTTATCATCCTTGGAGGGATGCTCTCCTATGCTTCCATATTTCTCTTTGGCCTTGAT      1620
GGATCCTTTGTCTCTGAAAAGACCTTTGAAACACTTTGCACCGTCAGGACCTGGATTCTC      1680
ACCGTGGGCTACACGACCGCTTTTGGGGCCATGTTTGCAAAGACCTGGAGAGTCCACGCC      1740
ATCTTCAAAAATGTGAAAATGAAGAAGAAGATCATCAAGGACCAGAAACTGCTTGTGATC      1800
GTGGGGGGCATGCTGCTGATCGACCTGTGTATCCTGATCTGCTGGCAGGCTGTGGACCCC      1860
CTGCGAAGGACAGTGGAGAAGTACAGCATGGAGCCGGACCCAGCAGGACGGGATATCTCC      1920
ATCCGCCCTCTCCTGGAGCACTGTGAGAACACCCATATGACCATCTGGCTTGGCATCGTC      1980
TATGCCTACAAGGGACTTCTCATGTTGTTCGGTTGTTTCTTAGCTTGGGAGACCCGCAAC      2040
GTCAGCATCCCCGCACTCAACGACAGCAAGTACATCGGGATGAGTGTCTACAACGTGGGG      2100
ATCATGTGCATCATCGGGGCCGCTGTCTCCTTCCTGACCCGGGACCAGCCCAATGTGCAG      2160
TTCTGCATCGTGGCTCTGGTCATCATCTTCTGCAGCACCATCACCCTCTGCCTGGTATTC      2220
GTGCCGAAGCTCATCACCCTGAGAACAAACCCAGATGCAGCAACGCAGAACAGGCGATTC      2280
CAGTTCACTCAGAATCAGAAGAAAGAAGATTCTAAAACGTCCACCTCGGTCACCAGTGTG      2340
AACCAAGCCAGCACATCCCGCCTGGAGGGCCTACAGTCAGAAAACCATCGCCTGCGAATG      2400
AAGATCACAGAGCTGGATAAAGACTTGGAAGAGGTCACCATGCAGCTGCAGGACACACCA      2460
GAAAAGACCACCTACATTAAACAGAACCACTACCAAGAGCTCAATGACATCCTCAACCTG      2520
GGAAACTTCACTGAGAGCACAGATGGAGGAAAGGCCATTTTAAAAAATCACCTCGATCAA      2580
AATCCCCAGCTACAGTGGAACACAACAGAGCCCTCTCGAACATGCAAAGATCCTATAGAA      2640
GATATAAACTCTCCAGAACACATCCAGCGTCGGCTGTCCCTCCAGCTCCCCATCCTCCAC      2700
CACGCCTACCTCCCATCCATCGGAGGCGTGGACGCCAGCTGTGTCAGCCCCTGCGTCAGC      2760
CCCACCGCCAGCCCCCGCCACAGACATGTGCCACCCTCCTTCCGAGTCATGGTCTCGGGC      2820
CTGTAA
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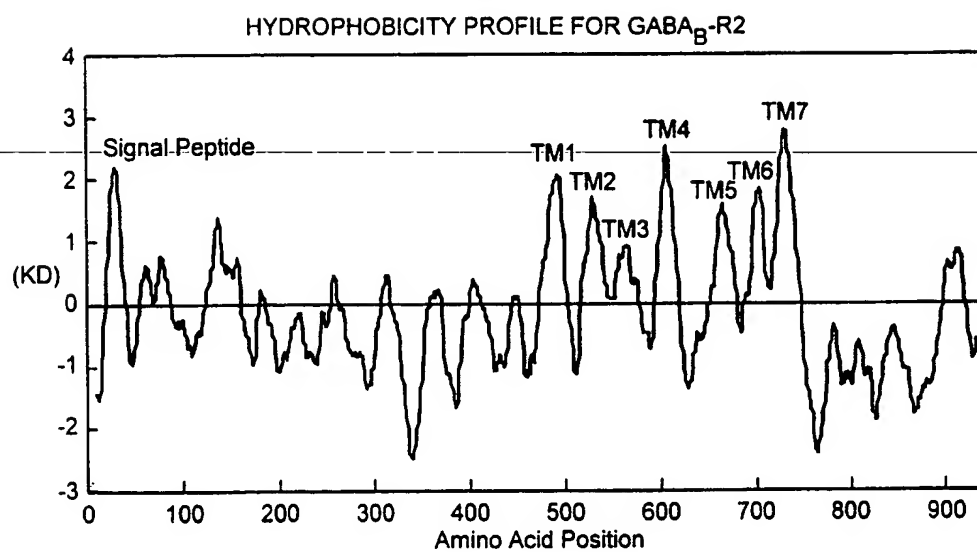
B. Protein Sequence

```
MASPRSSGQPGPPPPPPPPPPARLLLLLLLLPLLLPLAPGAWGWARGAPRPPPSSPPLSIMG        60
LMPLTKEVAKGSIGRGVLPAVELAIEQIRNESLLRPYFLDLRLYDTECDNAKGLKAFYDA         120
IKYGPNHLMVFGGVCPSVTSIIAESLQGWNLVQLSFAATTPVLADKKKYPYFFRTVPSDN         180
AVNPAILKLLKHYQWKRVGTLTQDVQRFSEVRNDLTGVLYGEDIEISDTESFSNDPCTSV         240
KKLKGNDVRIILGQFDQNMAAKVFCCAYEENMYGSKYQWIIPGWYEPSWWEQVHTEANSS         300
RCLRKNLLAAMEGYIGVDFEPLSSKQIKTISGKTPQQYEREYNNKRSGVGPSKFHGYAYD         360
GIWVIAKTLQRAMETLHASSRHQRIQDFNYTDHTLGRIILNAMNETNFFGVTGQVVFRNG         420
ERMGTIKFTQFQDSREVKVGEYNAVADTLEIINDTIRFQGSEPPKDKTIILEQLRKISLP         480
LYSILSALTILGMIMASAFLFFNIKNRNQKLIKMSSPYMNNLIILGGMLSYASIFLFGLD         540
GSFVSEKTFETLCTVRTWILTVGYTTAFGAMFAKTWRVHAIFKNVKMKKKIIKDQKLLVI         600
VGGMLLIDLCILICWQAVDPLRRTVEKYSMEPDPAGRDISIRPLLEHCENTHMTIWLGIV         660
YAYKGLLMLFGCFLAWETRNVSIPALNDSKYIGMSVYNVGIMCIIGAAVSFLTRDQPNVQ         720
FCIVALVIIFCSTITLCLVFVPKLITLRTNPDAATQNRRFQFTQNQKKEDSKTSTSVTSV         780
NQASTSRLEGLQSENHRLRMKITELDKDLEEVTMQLQDTPEKTTYIKQNHYQELNDILNL         840
GNFTESTDGGKAILKNHLDQNPQLQWNTTEPSRTCKDPIEDINSPEHIQRRLSLQLPILH         900
HAYLPSIGGVDASCVSPCVSPTASPRHRHVPPSFRVMVSGL                           941
```

FIG. 1 CONT'D

FIG. 2

GABAb1a   MLLLLLAPLFLRPPGA X
GABAb1b   MGPGAPFARVGWPLPLLVVMAAGVAPVWA ....
GABAb2   MASPRSSGQPGPPPPPPPPARLLLLLLLLPLLLPLAPG ....   GGAQTPNATSEGCQIIHPPWEGGIRYRGLTRDQVKAINFLPVDYEIEYVCRGEREV

GABAb1a   VGPKVRKCLANGSWTDMDTPSRCV|RICSKSYLTLENGKVFLTGGDLPALDGARVDFRCDPDFHLVGSSRSICSQGQWSTPKPHCQVNRTPH|
GABAb1a/b   SERRAVYIG
GABAb1b   SHSPHLPRPHSRVPPHPS
GABAb2   AWGWARGAPRPPPSSPPLS...IMGLM

GABAb1   ALFPMSGGWPGGQACQPAVEMALEDVNSRRDILPDYELKLIHHDSKCDPGQATKYLYELLYNDPIKIILMPG.CSSVSTLVAEAARMWNLIVLSYGSSSPA
GABAb2   PLTKEVAKGSIGRGVLPAVELAIEQIRN.ESLLRPYFLDLRLYDTECDNAKGLKAFYDAIKYGPNHLMVFGGVCPSVTSIIAESLQGWNLVQLSFAATTPV

GABAb1   LSNRQRFPTFFRTHPSATLHRPTRVKLFEKWGWKKIATIQQTTEVFTSTLDDLEERVKEAGIEITFRQSFFSDPAVPVKNLKRQDARIIVGLFYETEARKV
GABAb2   LADKKKYPYFFRTVPSDNAVNPAILKLLKHYQWKRVGTLTQDVQRFSEVRNDLTGVLYGEDIEISDTESFSNDPCTSVKKLKGNDVRIILGQFDQNMAAKV

GABAb1   FCEVYKERLFGKKYVWFLIGWYADNWFKIYDPSIN...CTVDEMTEAVEGHITTEIVMLNPANTRSISNMTSQEFV.EKLTKRLKRHPEETGGFQEAPLAX
GABAb2   FCCAYEENMYGSKYQWIIPGWYEPSWWEQVHTEANSSRCLRKNLLAAMEGYIGVDFEPLSSKQIKTISGKTPQQYEREYNNKRSGVGPSKFHGY.....AY

GABAb1   DAIWALALALNKTSGGGGRSG..VRLEDFNYNNQTITDQIYRAMNSSSFEGVSGHVVFDASGSRMAWTLIEQPQGGSYKKIGYDSTKDDLS.WSKTDKWIG
GABAb2   DGIWVIAKTLQRAMETLHASSRHQRIQDFNYTDHTLGRIILNAMNETNFFGVTGQVVF.RNGERMG.TIKFTQFQDSREVKVGEYNAVADTLEIINDTIRFQ

GABAb1   GSPPA.DQTLVIKTFRFLSQKLFISVSVLSSLGIVLAVVCLSFNIYNSHVRYIQNSQPNLNNLTAVGCSLALAAVFPLGLDGYHIGRNQFPFVCQARLWLLG
GABAb2   GSEPPKDKTIILEQLRKISLPLYSILSALTILGMIMASAFLFTNIKNRNQKIKMSSPYMNNLIILGGMLSYASIFLFGLDGSFVSEKTFETLCTVRTWILT
                TMI   TMII

GABAb1   LGFSLGYGSMETKIWWVHTGFTKKEEKWRKTLEPWKLYATVGLLVGMDVLTLAINQIVDPLHRTIETFAKEEPKEDIDVSILPQLEHCSSRKMNTWLGIF
GABAb2   VGYTTAFGAMFAKTWRVHAIFKNVKMKKKIIKDQK...LLVIVGGMLLIDLCILCIWQAVDPLRRTVEKYSMEPDPAGRDISIRPLLEHCENTHMTIWLGIV
                TMIII   TMIV   BAIT →

GABAb1   YGYKGLLLLLGIFLAYETKSVSTEKINDHRAVGMAIYNVAVLCLITAPVTMLLSSQQDAAFAFASLAIVFSSYITLVVLFVPKMRRLITRGE.........
GABAb2   YAYKGLLMLFGCFLAWETRNVSIPALNDSKYIGMSVYNVGIMCIIGAAVSFLTRDQPNVQFCIVALVIIFCSTITLCLVFVPK...LITLRTNPDAATQNRR
                TMV   TMVI   TMVII

GABAb1   WQSEA.QDTMKTGSSTNNEEEK.SRLLEK.EMRELELKIAEKEERVSELRHQLQSRQQLRSRRHPPTPPEPSGGLPRGPPEPPDRLSCDGSRVHLLYK
GABAb2   FQFTQNQKKEDSKTSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKDLEEVTMQLQDTPEKTTYIKQNHYQELNDILNLGNFTESTDGGKAILKNHLDQN
           Y2H HITS →

GABAb2   PQLQWNTTEPSRTCKDPIEDINSPEHIQRRLSLQLPILHHAYLPSIGGVDASCVSPCVSPTASPRHRHVPPSFRVMVSGL

FIG. 3

HYDROPHOBICITY PROFILE FOR GABA$_B$-R2

FIG. 4A

GABA$_B$-R1 DISTRIBUTION

Whole Brain	Amygdala	Caudate nucleus	Cerebellum	Cerebral Cortex	Frontal lobe	Hippocampus	Medulla oblongata

Occipital lobe	Putamen	Substantia nigra	Temporal lobe	Thalamus	Subthalamic nucleus	Spinal cord

Heart	Aorta	Skeletal muscle	Colon	Bladder	Uterus	Prostate	Stomach

Testis	Ovary	Pancreas	Pituitary gland	Adrenal gland	Thyroid gland	Mammary gland	Salivary gland

Kidney	Liver	Small intestine	Spleen	Thymus	Peripheral leukocyte	Lymph node	Bone marrow

Appendix	Lung	Trachea	Placenta

Fetal brain	Fetal heart	Fetal kidney	Fetal liver	Fetal spleen	Fetal thymus	Fetal lung

GABA$_B$-R1 TISSUE DISTRIBUTION

FIG. 4B

GABA$_B$-R2 DISTRIBUTION
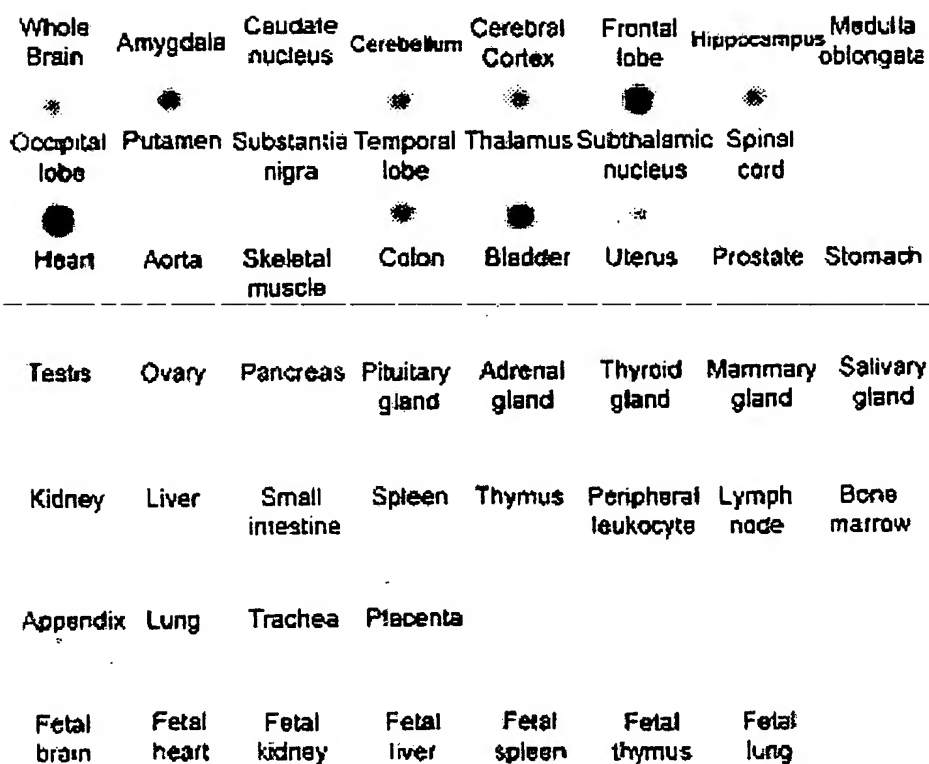
GABA$_B$-R2 TISSUE DISTRIBUTION

FIG. 5

HETERODIMERSATION AND HOMODIMERISATION BETWEEN THE
C-TERMINAL DOMAINS OF THE $GABA_B$-R1 AND $GABA_B$-R2 RECEPTORS
IN THE YEAST TWO HYBRID SYSTEM.
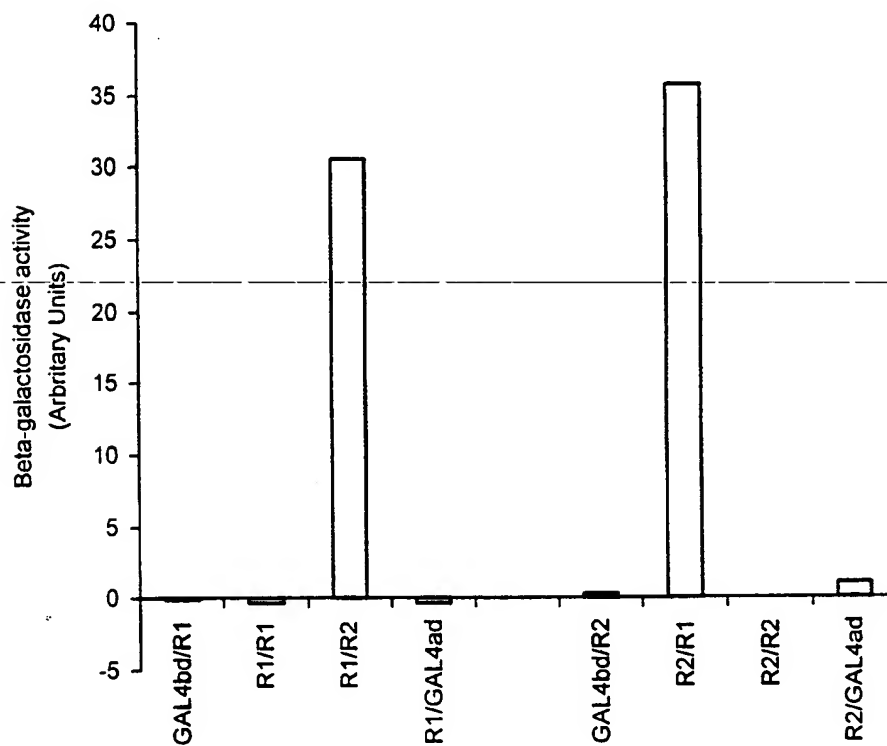
FIG. 6

CO-IMMUNOPRECIPITATION STUDIES OF THE GABA$_B$
HETERODIMER IN HEK239 CELLS
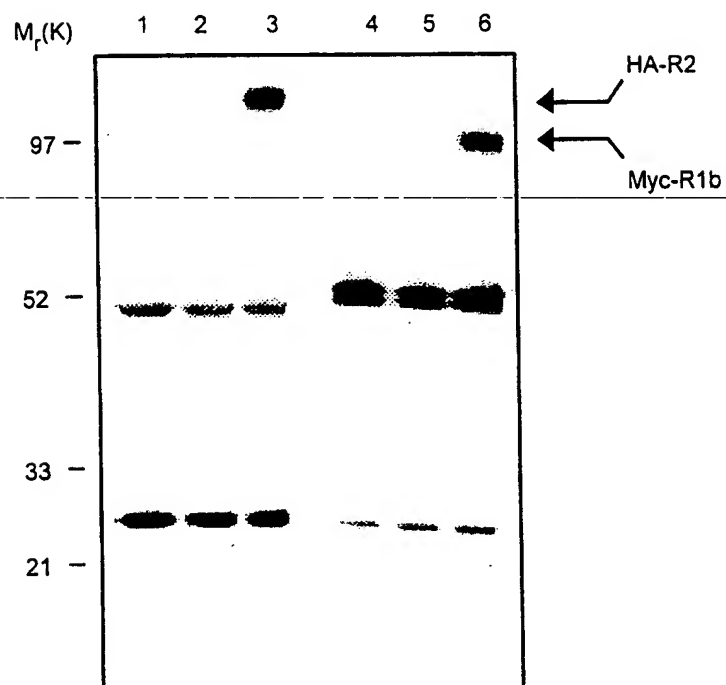
FIG. 7

CELL SURFACE LOCALISATION OF GABA$_B$-R1b RECEPTOR IS
DEPENDANT UPON CO-EXPRESSION WITH GABA$_B$-R2
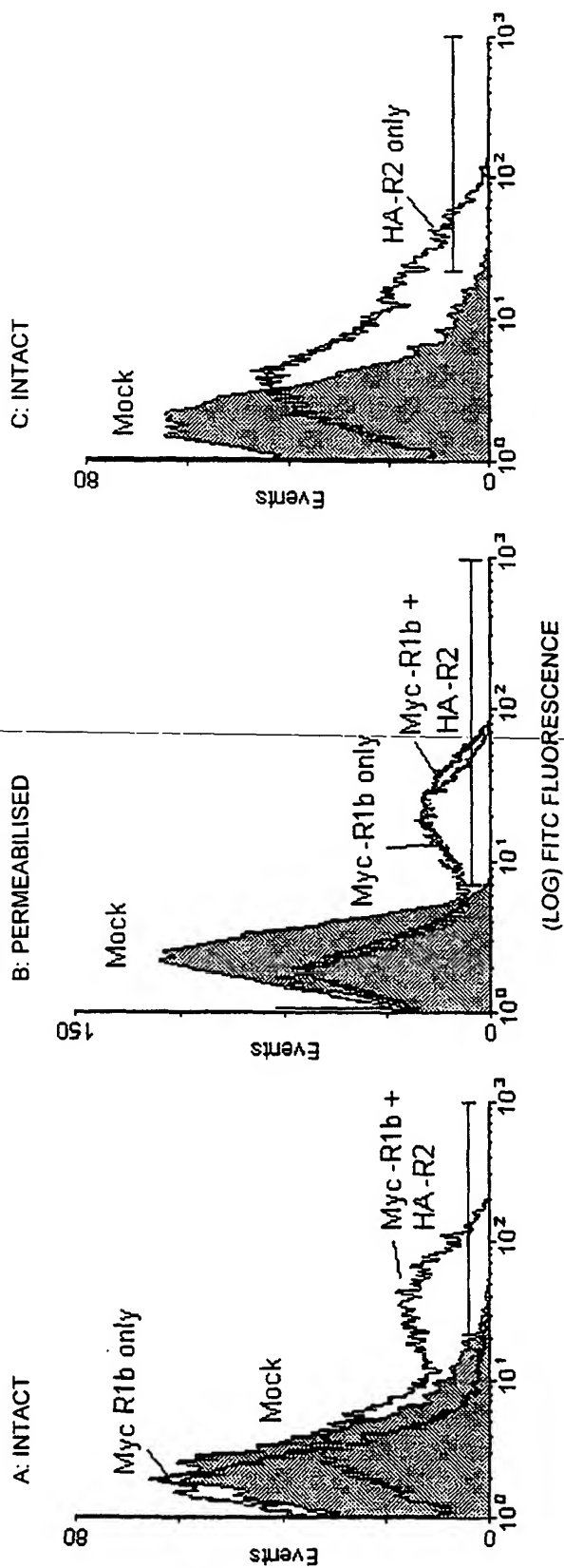
FIG. 8

COEXPRESSION OF GABA$_B$-R1a AND 1b SPLICE VARIANTS WITH
GABA$_B$-R2 RECEPTORS IN HEK293T CELLS RESULTS IN TERMINAL
GLYCOSYLATION OF BOTH GABA$_B$-R1a AND 1b

	R1a			R1b			HA-R2+R1a			HA-R2+R1b	
LANE	1	2	3	4	5	6	7	8	9	10	11	12

ENZYME   -   F   H   -   F   H   -   F   H   -   F   H

	HA-R2			HA-R2+R1a			HA-R2+R1b	
LANE	13	14	15	16	17	18	19	20	21

ENZYME   -   F   H   -   F   H   -   F   H
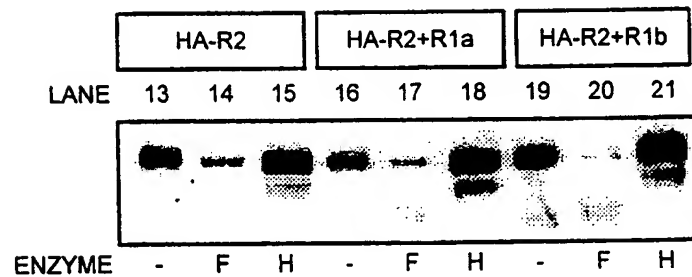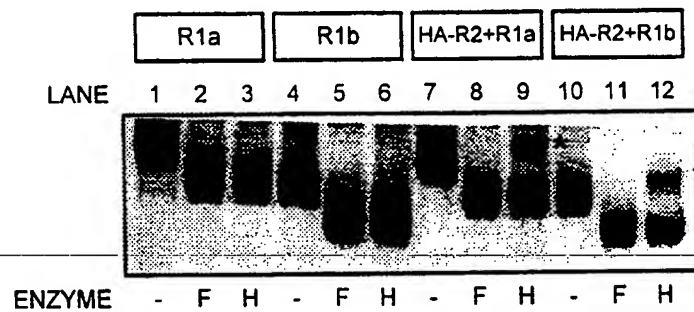
FIG. 9A

COEXPRESSION OF GABA$_B$-R1 AND GABA$_B$-R2 RECEPTORS
IN HEK293T CELLS LEADS TO GABA-MEDIATED STIMULATION
OF [^{35}S]GTPγS BINDING ACTIVITY
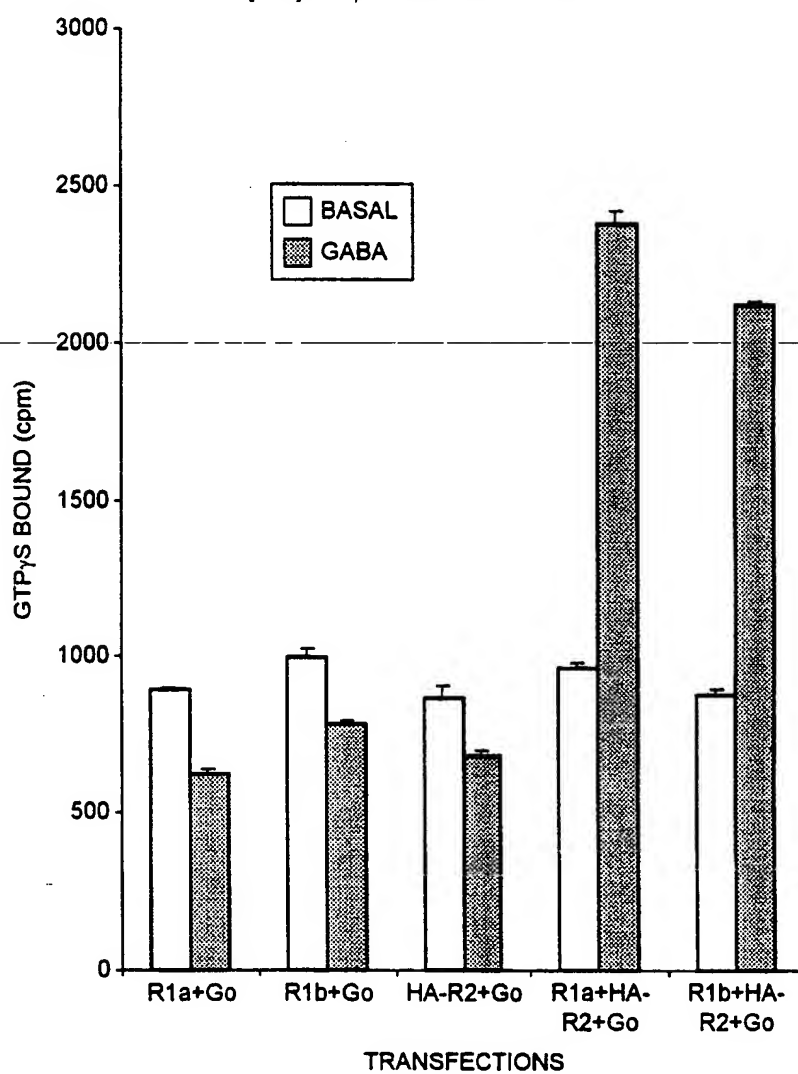
FIG. 9B

COEXPRESSION OF $GABA_B$-R1 AND $GABA_B$-R2 RECEPTORS IN
HEK293T CELLS LEADS TO GABA-MEDIATED STIMULATION OF
$[^{35}S]GTP\gamma S$ BINDING ACTIVITY IN A DOSE DEPENDANT MANNER.
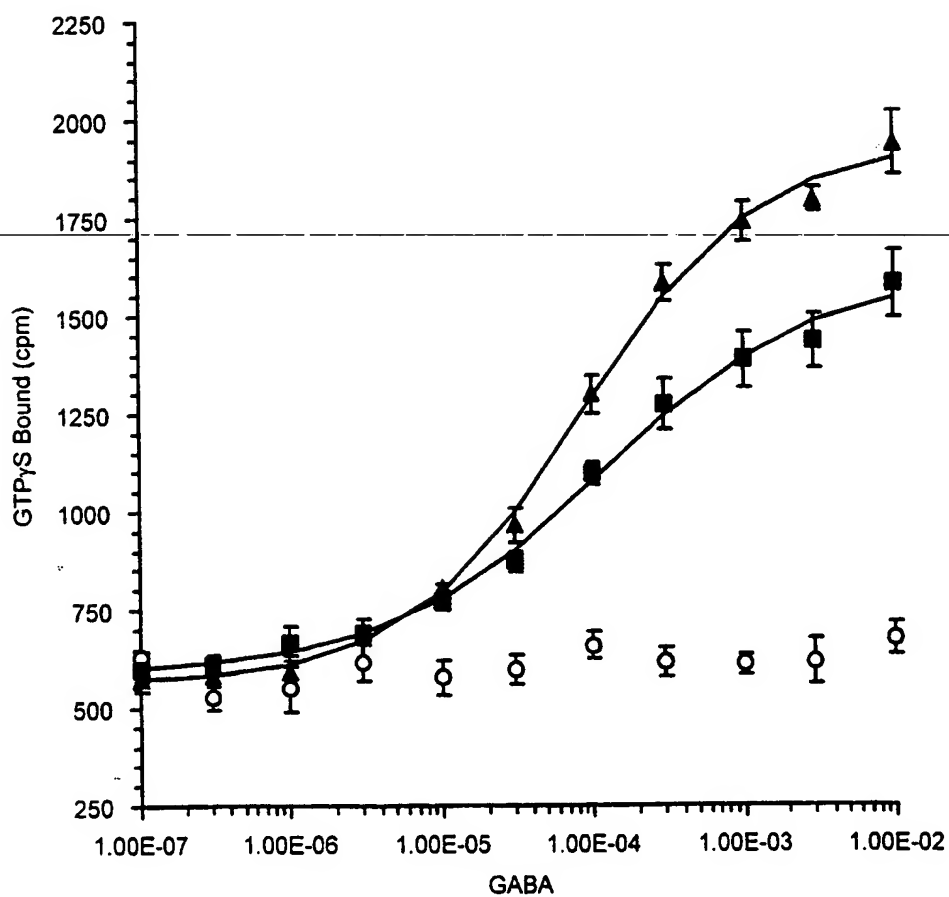
FIG. 10

GABA-MEDIATED STIMULATION OF[^{35}S]GTPγS BINDING ACTIVITY IN
HEK293T CELLS COEXPRESSING GABA$_B$-R1 AND GABA$_B$-R2 RECEPTORS
REQUIRES COTRANSFECTION WITH ADDITIONAL G$_i$ G-PROTEIN, G$_{o1}$α.
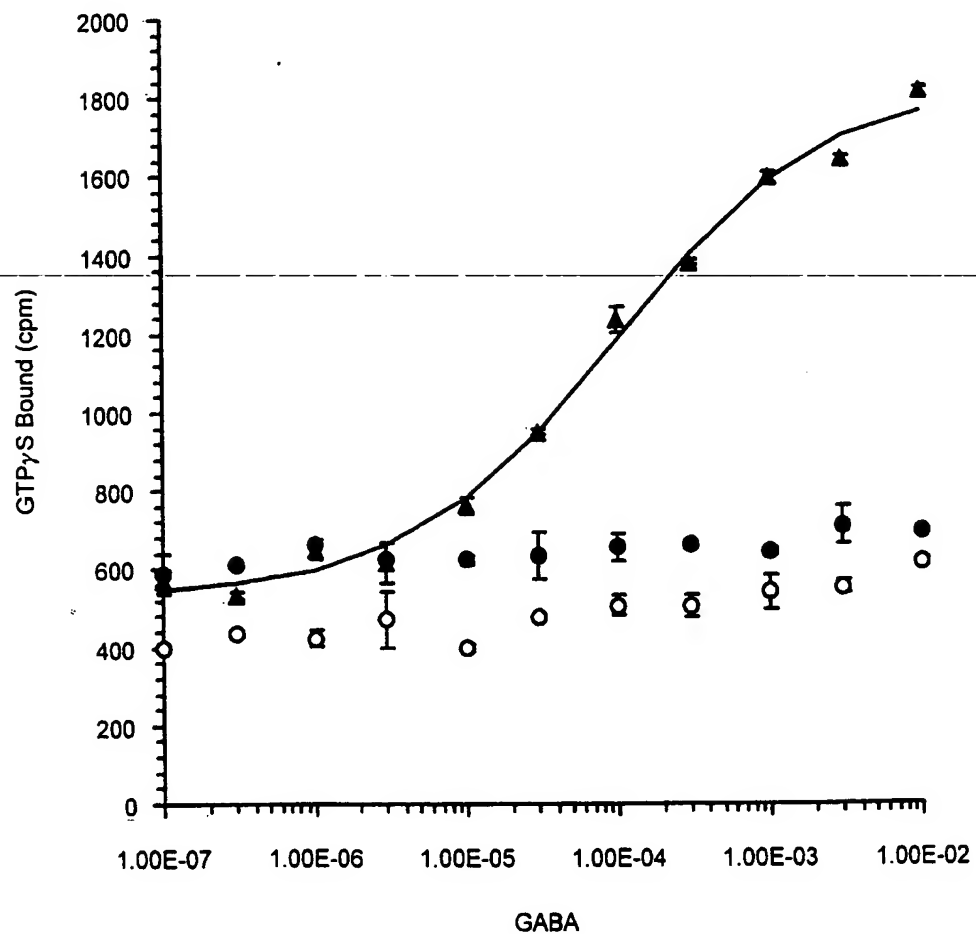
FIG. 11A

COEXPRESSION OF GABA$_B$-R1 AND GABA$_B$-R2 RECEPTORS IN
HEK293T CELLS PERMIT GABA-MEDIATED INHIBITION OF FORSKOLIN-
STIMULATION ADENYLATE CYCLASE ACTIVITY
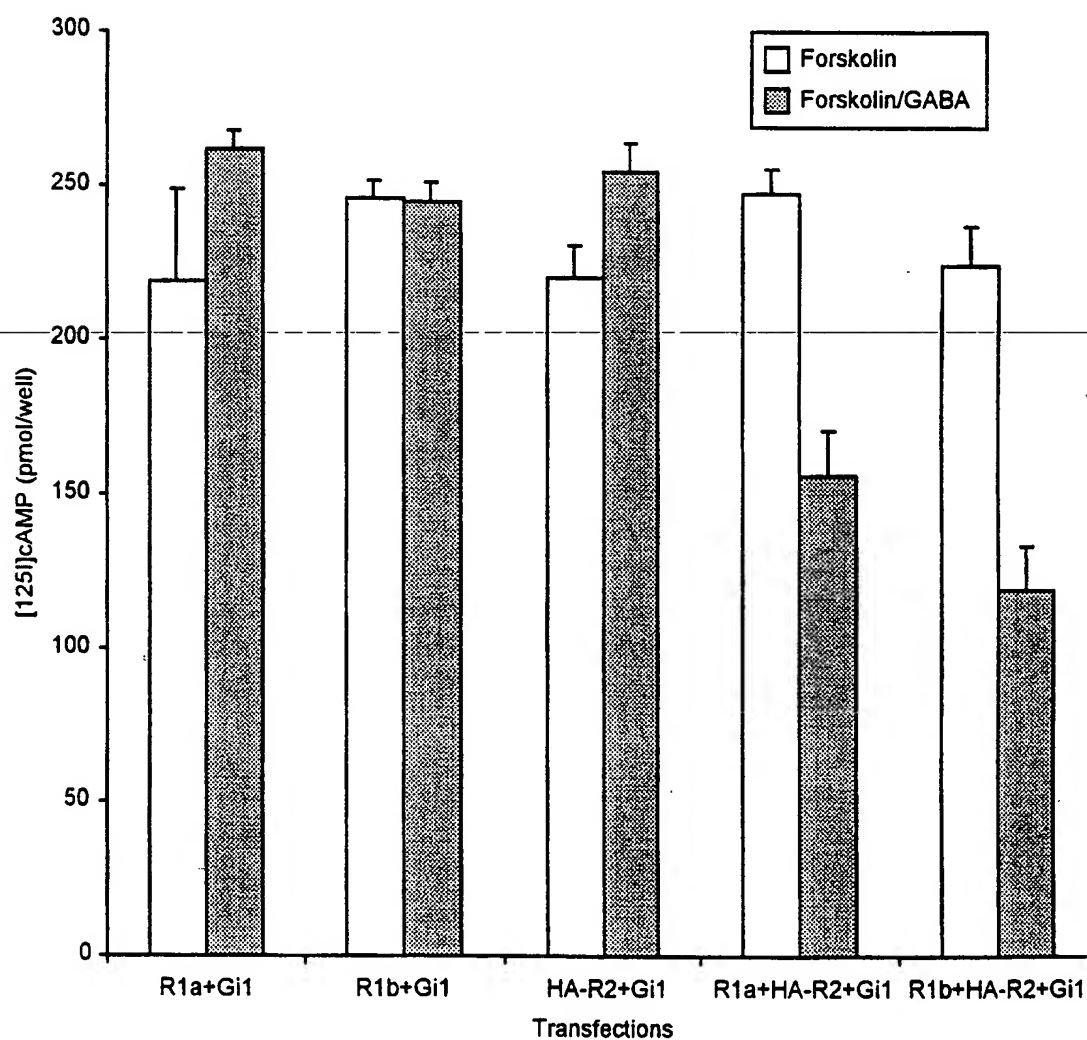
FIG. 11B

COEXPRESSION OF GABA$_B$-R1 AND GABA$_B$-R2 RECEPTORS IN
HEK293T CELLS PERMITS GABA-MEDIATED INHIBITION OF
FORSKOLIN-STIMULATED ADENYLATE CYCLASE ACTIVITY IN A
DOSE DEPENDANT MANNER
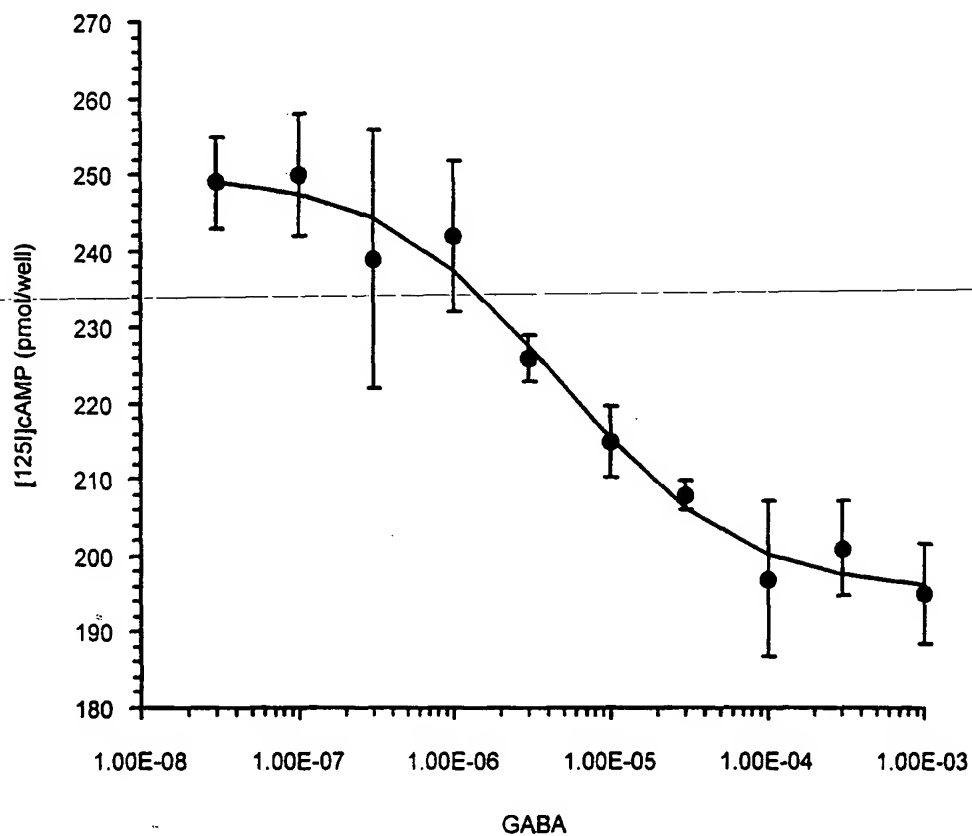
FIG. 12A

CO-EXPRESSION OF GABA$_B$-R1 AND GABA$_B$-R2 RECEPTORS IN OOCYTES LEADS TO AGONIST-DEPENDANT ACTIVATION OF ION FLUX THROUGH CFTR
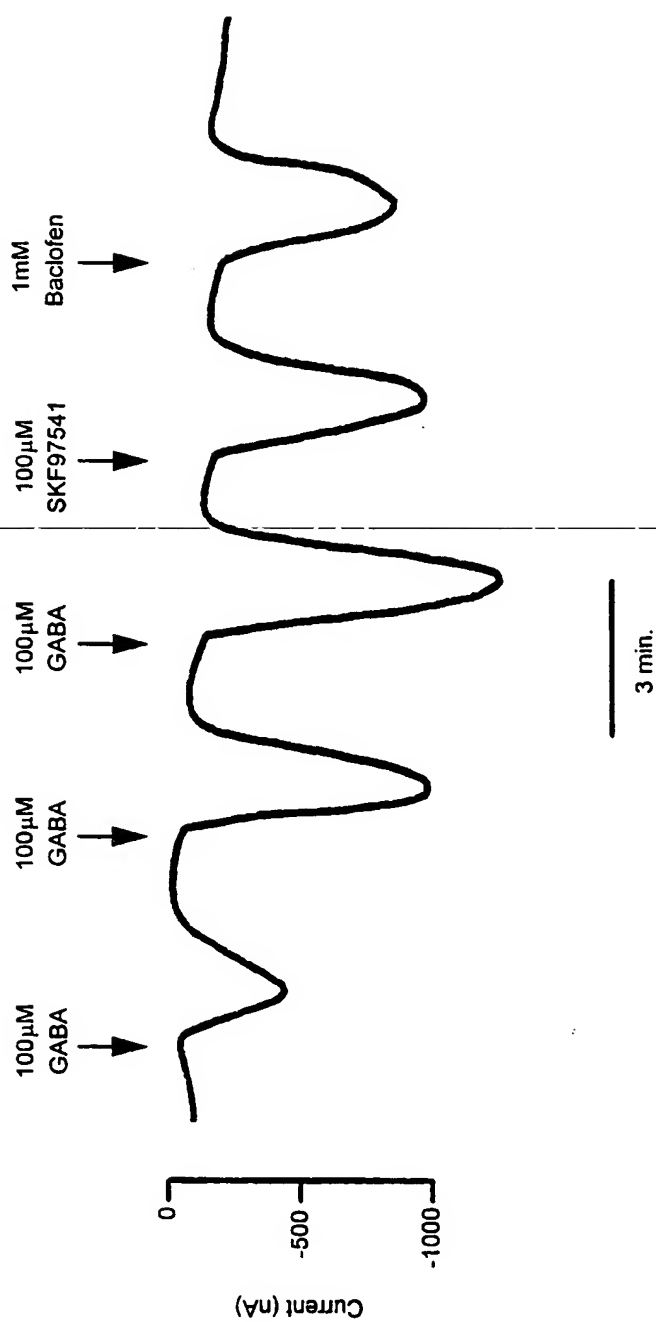
FIG. 12B

CO-EXPRESSION OF GABA$_B$-R1 AND GABA$_B$-R2 IN OOCYTES
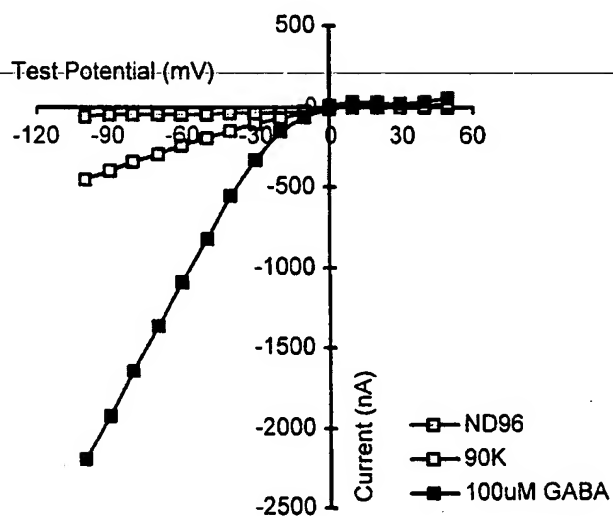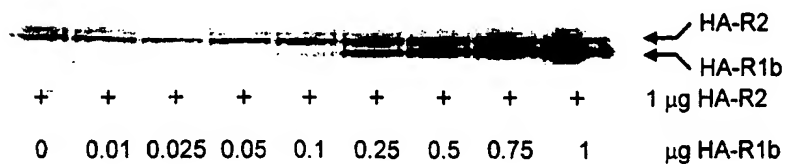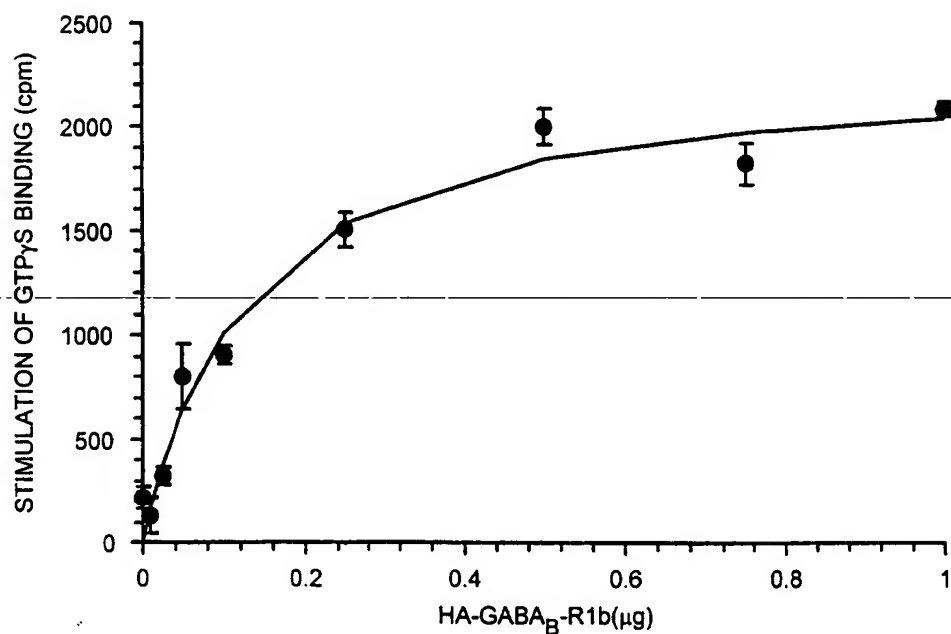LEADS TO AGONIST ACTIVATION OF ION FLUX THROUGH GIRK
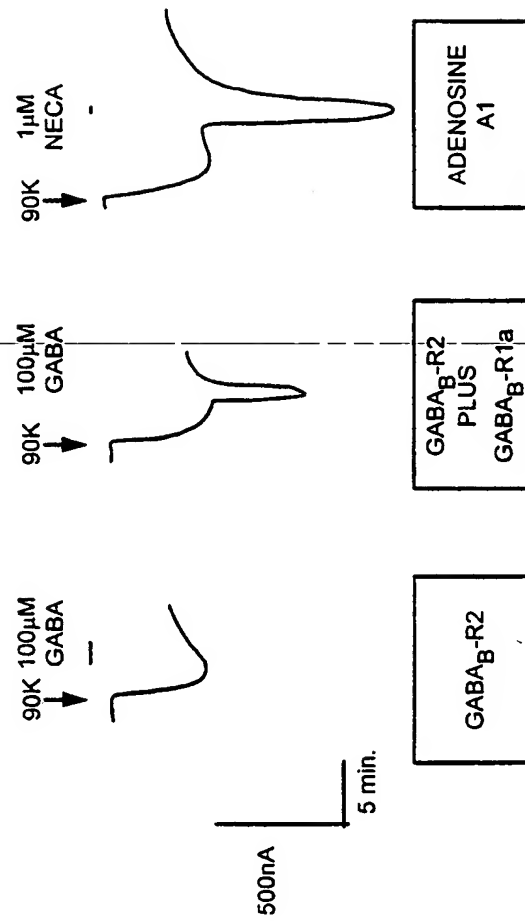
FIG. 13

CURRENT-VOLTAGE CURVES IN AN OOCYTE EXPRESSING GABA$_B$-R1, GABA$_B$-R2 AND THE POTASSIUM CHANNELS GIRK1 AND GIRK4

FIG. 14

GABA-MEDIATED STIMULATION OF[^{35}S]GTPγS BINDING ACTIVITY IS
DEPENDENT ON THE RELATIVE LEVELS OF EXPRESSION OF
GABA$_B$-R1 AND GABA$_B$-R2 RECEPTORS

FIG. 15

CO-EXPRESSION OF $GABA_B$-R1 AND $GABA_B$-R2 RECEPTORS IN HEK293T
CELLS GENERATES A HIGH AFFINITY $GABA_B$ BINDING SITE SIMILAR TO
BRAIN $GABA_B$ RECEPTORS